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ORIGINAL PAPER

Preparation of Diacid 1,3-Diacylglycerols

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Abstract A complete methodology (including synthesis, purification and analysis) for the preparation of 1,3-DAG is described. For a successful synthesis project, the strengths and weaknesses of each particular process should be taken into account and measures taken to offset or balance potential weaknesses. To this end, we describe some of the challenges associated with: chemically and enzymatically catalyzed acylglycerol syntheses; recrystallization and flash chromatography for purification of partial acylglycerols; and thin-layer chromatography (TLC) separation of DAG. For this work, 1-MAG intermediates and subsequent diacid 1,3-DAG were prepared using non-enzymatic methods, whereas, monoacid 1,3-DAG were prepared by enzymatic methods. It was not always possible to obtain pure samples of target compounds—in recrystallizations this is due to solid solution formation and co-crystallization and in chromatographic separations it is due to co-elution of components with similar R_f. Furthermore, TLC R_f of DAG is determined by two main factors: acyl chain length and positional isomerism. Interestingly, while the role of positional isomerism is well-known, the role of acyl chain length in these separations has only recently come to light.

Keywords Acylglycerol synthesis · Esterification · 1,3-Diacylglycerols · Thin-layer chromatography · Flash chromatography · Acylglycerol analysis

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Introduction

A great deal of information can be garnered from the study of natural systems, which are by and large complex and multi-component. However, at some stage all scientific inquiry requires the examination of simpler systems without the interference of extraneous material or variables. Examples from fats and oils include: the investigation of lipase selectivity and specificity [1]; investigations of chemical, thermodynamic and analytical parameters for pure TAG [2]; and food product development. Simple monoacid acylglycerols of high purity are now available for purchase and it may be convenient to use them in model systems. However, these compounds are often poor substitutes for the components in the natural system and, consequently, provide models that are not truly representative. When this is the case, molecules that are more reflective of the system's true chemistry should be prepared.

The chemical reactions used to prepare acylglycerols perform two basic functions, they either form covalent bonds between a FA and glycerol (i.e., esterification or acylation) or they block esterification at one or more sites to create or maintain positional specificity (i.e. protection and deprotection). Reactants in acylation reactions are an acyl donor (e.g., FA, acid chloride, acid anhydride) and glycerol or a glycerol derivative. Protected derivatives of glycerol include: isopropylidene-glycerol (solketal), which leaves a terminal hydroxyl available; benzylidene-glycerol, which leaves the middle hydroxyl available; and trityl-glycerol, which can block one or more hydroxyl group in any position. There are many excellent reviews of these chemically catalyzed acylation reactions and related approaches [3–7].

Enzymatic synthesis is attractive because regioselective lipases combine esterification and positional specificity in one synthetic step. Enzymatic esterification also requires

less vigorous conditions, making it a more appropriate choice for syntheses with PUFA. Several good reviews of enzymatic approaches to acylglycerol synthesis are available [8, 9].

While it is often given the highest profile, the synthetic method is only one of the critical components in a successful synthetic program. To quote Robert Jensen: "Synthesis is an art because, while it is not a problem to follow directions and mix reactants, recovery of the desired glyceride can be extremely difficult" [1]. In addition to the synthetic approach, equal consideration and resources should be afforded the subsequent purification and analysis of product. In this article, we relate our experiences and observations in the preparation of a series of 1,3-DAG. It was difficult to obtain all of the synthetic 1,3-DAG at sufficient purity for further applications. In light of this, we sought to understand the mechanisms by which the purity of these compounds was impaired. More importantly, we also sought to understand the means by which their purity in subsequent iterations may be improved.

Materials and Methods

Note: In the following sections references in angular brackets (i.e., ⟨1⟩) indicate the chemical species and reactants found in Fig. 1.

Unless noted otherwise: reagents, chemicals and enzyme were purchased from Sigma-Aldrich (Mississauga, ON) and were of the highest practical grade; solvents were purchased from Fisher Scientific (Ottawa, ON) and were HPLC grade. The declared purity of free fatty acids (FFA) was: hexanoic (6:0) and myristic (14:0) acids were 99.5%, palmitic (16:0) acid was ≥96%, lauric (12:0) acid was ≥95% and oleic (18:1) acid was ≥90% purity. The declared purity of acid chlorides was: butyroyl (4:0) and octanoyl (8:0) chloride were ≥99%, decanoyl (10:0), lauroyl and palmitoyl chloride were all 98% and hexanoyl chloride was 97% purity. Solketal (isopropylidene glycerol) and acetic anhydride were both 98% purity. The vinyl ester of palmitic acid donated by Japan VAM & POVAL (Osaka, JP) was ≥96% purity.

Monoacylglycerol Synthesis

The procedure for synthesizing monoacylglycerols ⟨4⟩ was based on those of Robert Jensen's group [6, 10] except solketal ⟨1⟩ was used as a starting material rather than glycerol (Fig. 1, reaction A) and much shorter reflux times were employed. To prepare 1(3)-monopalmitin, 32 g (0.124 mol) palmitic acid, 20 g (0.15 mol) solketal and 1 g *p*-toluenesulfonic acid (pTSA·H_2O) were combined with 250 mL toluene in a 1-L round-bottomed flask equipped with a 10 mL Dean–Stark trap, condenser, stirring bar and boiling beads. After a 4–5 h reflux, approximately 2.3 mL water had accumulated in the Dean–Stark trap. Once cool, the contents of the reaction flask were shaken with 1.0 g sodium acetate, washed three times with 100 mL of brine solution, dried over sodium sulfate, filtered, and the solvent was removed with a rotary evaporator.

Acetonide Cleavage

The free MAG was produced via an acetonide cleavage reaction [11]. 35 g of the condensation product ⟨3⟩ was refluxed for 3 h in a 500-mL round-bottomed flask containing 150 mL 95% ethanol and 3.5 g of Amberlyst™ 15 (wet). Once cool, the solvent was removed by rotary evaporation. To purify, MAG ⟨4⟩ was recrystallized from either acetone, hexane or hexane/diethyl ether (60:40 by volume).

Diacid 1,3-Diacylglycerol Synthesis

Monoacylglycerols were esterified with acid chlorides (FACl) ⟨5⟩ by following the procedure outlined by Gaffney and Reese [12] (Fig. 1, reaction B). For example, 16:0-OH-6:0 was prepared in the following manner: 10 g (0.03 mol) of monopalmitin, 4.6 mL of hexanoyl chloride (0.033 mol) and 0.36 g (0.003 mol) 4-dimethylaminopyridine (DMAP) were dissolved in 80 mL methylene chloride in a 500-mL round-bottom flask equipped with a magnetic stirrer. This solution was stirred in an ice bath while 7.5 mL (0.054 mol) of triethylamine (Et_3N) dissolved in 20 mL methylene chloride was added dropwise. Once the addition was complete, the ice bath was removed and the mixture was stirred for an additional 3 h at room temperature. Thin-layer chromatography (TLC) was used to monitor the reaction's progress. Once the reaction was complete, 1 mL of water was added to the flask and all solvent was removed by rotary evaporation. The residue was taken up in hexane and filtered, the filtrate was dried over sodium sulfate, filtered and the solvent evaporated. For some initial syntheses (6:0-OH-12:0 and 16:0-OH-12:0), pyridine was both base and catalyst [6, 10]; however, this method was discontinued in favor of the one already discussed.

An acid anhydride (FAOFA) ⟨7⟩ can be used in place of the acid chloride (Fig. 1, reaction C); for instance, acetic anhydride was used to produce 2:0-OH-16:0. 5 g (0.015 mol) monopalmitin, 1.56 mL (0.0165 mol) acetic anhydride and 0.18 g (0.0015 mol) DMAP dissolved in 40 mL methylene chloride in a 250-mL round-bottom flask equipped with magnetic stirrer. This solution was stirred in an ice bath while 3.75 mL (0.027 mol) triethylamine in

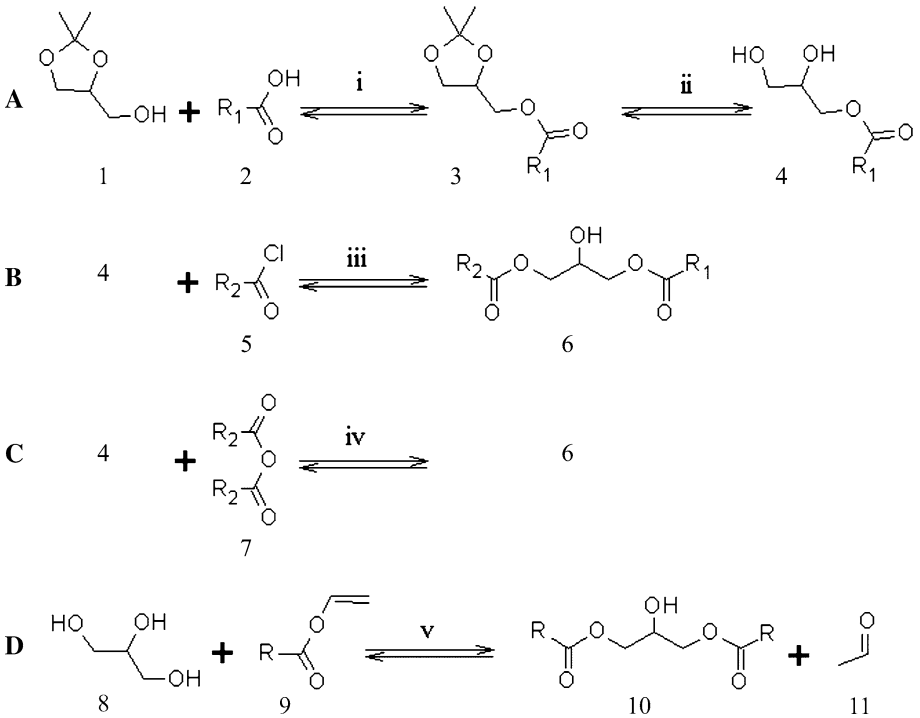

Fig. 1 Reaction scheme for 1,3-DAG synthesis: *Reactions:* **a** synthesis of monoacylglycerol; **b**, **c** synthesis of diacid 1,3-diacylglycerol with acid chloride and acid anhydride; **d** synthesis of monoacid 1,3-diacylglycerol. *Main chemical species:* ⟨*1*⟩ solketal; ⟨*2*⟩ fatty acid; ⟨*3*⟩ monoacylglycerol acetonide; ⟨*4*⟩ monoacylglycerol; ⟨*5*⟩ fatty acid chloride; ⟨*6*⟩ diacid 1,3-diacylglycerol; ⟨*7*⟩ acid anhydride; ⟨*8*⟩ glycerol; ⟨*9*⟩ vinyl ester; ⟨*10*⟩ monoacid 1,3-diacylglycerol; ⟨*11*⟩ acetaldehyde. *Reactants, catalysts and conditions:* (i) *p*-toluene sulfonic acid, reflux (Δ), Dean–Stark apparatus; (ii) Amberlyst™ 15 (wet) (Dow Chemical; Midland, MI), Δ; (iii), (iv) triethylamine, dimethylaminopyridine (DMAP), at room temperature (RT); (v) *Candida antarctica* lipase B, 0–4 °C

10 mL methylene chloride was added dropwise. The remainder of reaction was identical to description for 16:0-OH-6:0.

Pure 1,3-DAG were obtained by flash chromatography or by recrystallization from acetone, hexane/ethanol (8:2, v/v) or hexane with a minimum quantity of ethyl acetate.

Monoacid 1,3-Diacylglycerol Synthesis

Enzymatic synthesis was useful for producing monoacid DAG ⟨10⟩ starting from glycerol ⟨8⟩ and a fatty acid vinyl ester (FAVE) ⟨9⟩ (Fig. 1, reaction D) [13]. For example, 2.0 g of glycerol (0.0217 mol) was added to 13.2 mL (specific gravity 0.861; 15.3 g; 0.0543 mol) vinyl palmitate and 1.0 g of immobilized lipase B from *Candida antarctica* in 60 mL of methylene chloride stirred under a nitrogen atmosphere in an ice water bath. The reaction was monitored by TLC and after 3.75 h reaction was worked up. The solution was warmed to dissolve solids and the immobilized enzyme was filtered from the solution. Solvent was removed on a rotary evaporator and the product was recrystallized from hexane/ethyl acetate 97:3 (v/v).

Flash Chromatography

Samples were purified on a glass 2-in diameter column with a 500 mL reservoir (United Glass Technologies, Philadelphia, PA, USA) packed with 6–8 in. (approximately 200 g) of silica gel. Column loading should not exceed 1 g and each packed column should only be used once. Flash chromatography was conducted using plain silica gel (grade 60; 230–400 mesh) (Fisher Scientific) or boric acid-treated silica gel. Silica gel is often pretreated with boric acid to minimize acyl migration during TLC separations [14–16] and this has also been recommended for separating partial acylglycerols by flash chromatography [17]. We found no appreciable advantage to using boric acid-treated silica gel and since it was time consuming to prepare and difficult to handle (tended to clump), we discontinued its use.

The method for flash chromatography as described in the original paper was followed to isolate most products [18]. For flash chromatography, a hexane/ethyl acetate blend that provides a retention factor (R_f) of 0.35 (by TLC) for the component of interest is considered optimal. For example, 6:0-OH-16:0 was isolated using hexane/ethyl acetate (4:1 by volume) since this solvent produced a TLC R_f of 0.36.

Thin-Layer Chromatography

Glass-backed TLC plates were required because they can withstand the charring procedure used for visualization. Large 20 × 20 cm plates with a 250-μm layer of silica G (Analtech Inc., Newark DE) and small 2.5 × 7.5 cm with a 250-μm layer of silica gel 60 and fluorescent indicator (Merck, Darmstadt, DE) were used. Large plates were used to determine 1,3-DAG R_f values whereas small plates were used primarily to monitor separations and reactions. Boric acid treated TLC plates were prepared by spraying commercial glass-backed plates with a saturated solution of boric acid (~15%) in water/methanol (25:75, by volume) and drying in a 120 °C oven for 30 min [14, 16]. Untreated plates were activated by heating in an oven (~100 °C) for 30 min.

Plates were developed using either chloroform/acetone (96/4; by volume) or hexane/ethyl acetate (various ratios). Chloroform/acetone was used with boric acid treated plates for isolation of 1,2- from 1,3-DAG and 2- from 1(3)-MAG since boric acid hinders acyl migration [16]. Hexane/ethyl acetate was often used to assess or monitor isolation of compounds by flash chromatography.

Compounds were visualized by charring plates that had been dipped in a *p*-anisaldehyde solution. While a number of methods are used for the visualization of acylglycerols on TLC plates (e.g., iodine, primuline, dichlorofluorescein), most of these methods are ineffective in the analysis of compounds that do not contain unsaturated FA. Dipping in *p*-anisaldehyde solution followed by charring was the only reliable method for rapid visualization of saturates. The *p*-anisaldehyde solution was prepared by combining 135 mL 95% ethanol, 5 mL sulfuric acid (conc.), 3.7 mL *p*-anisaldehyde and 15 mL glacial acetic acid while stirring in an ice bath (verbal communication with Professor Adrian Schwan, Department of Chemistry, University of Guelph). This method worked best when small TLC plates were dipped and quickly removed from the solution then left on a paper towel for ~1 min to allow some of the solvent to evaporate. Afterwards, the plates were heated on a hot plate (set at ~200 °C). Large (20 × 20 cm) plates were charred by spraying thoroughly with *p*-anisaldehyde solution and, once dry, heating in an oven (vented to a fume hood) at ~160 °C.

Nuclear Magnetic Resonance

Approximately 15 mg of the sample dissolved in 750 μL deuterated chloroform (containing 0.5% tetramethylsilane (TMS) as internal standard) was placed in a suitable NMR

Table 1 Summary of methods and product purity by gas chromatography

Compound		R_1	R_2	Reaction	Purification[a]	Solvent[b]	Purity (%)	Yield[c] (%)
1(3)-Monoacylglycerols								
	12:0-OH-OH	12:0	–	A	R	hex./Et_2O (4:1)		
	14:0-OH-OH	14:0	–	A	R	hex./EtOAc (~97:3)		
	16:0-OH-OH	16:0	–	A	R	Acetone, hex/Et_2O (4:1)		
	18:1-OH-OH	18:1	–	A	R	hex./Et_2O (4:1)		
1,3-Diacylglycerols								
A	6:0-OH-12:0	12:0	6:0	B[d]	FC	hex./EtOAc (3:1)	>99.0	50
B	6:0-OH-16:0	16:0	6:0	B	FC	hex./EtOAc (4:1)	86.05	60
C	6:0-OH-18:1	18:1	6:0	B	FC	hex./EtOAc (5:1)	75.28	60
D	12:0-OH-16:0	16:0	12:0	B[d]	R	hex./EtOAc (4:1)	>99.0	45
E	12:0-OH-18:1	18:1	12:0	B	FC	hex./EtOAc (4:1)	94.25	30
F	18:1-OH-16:0	16:0	18:1	B	FC	hex./EtOAc (4:1)	95.22	46
G	2:0-OH-16:0	16:0	2:0	C	FC	hex./EtOAc (5:2)	84.97	21.5
H	4:0-OH-16:0	16:0	4:0	B	FC	hex./EtOAc (3:1)	92.94	24.0
I	8:0-OH-16:0	16:0	8:0	B	FC	hex./EtOAc (3:1)	91.63	51
J	10:0-OH-16:0	16:0	10:0	B	FC	hex./EtOAc (7:2)	73.98	62
K	14:0-OH-16:0	14:0	16:0	B	R	hex./EtOAc (~97:3)	72.62	
L	16:0-OH-16:0	16:0	–	D	R	hex./EtOAc (~97:3)	>99.0	35

R_1, R_2 and Reaction refer to features noted in Fig. 1

[a] *FC* flash chromatography, *R* recrystallization

[b] Solvent ratios are by volume; *hex*. hexane, *Et_2O* diethyl ether, *EtOAc* ethyl acetate

[c] Approximate, not optimized

[d] Pyridine instead of Et_3N and DMAP

tube (Wilmad, Buena, NJ). Proton NMR of samples was obtained using an Avance III 400 MHz instrument (Bruker, Billerica, MA). Chemical shifts were measured relative to tetramethylsilane (internal standard). NMR spectra were integrated and analyzed using Topspin 2.1 software (Bruker).

Characteristic chemical shifts were determined by referring to a number of publications: peaks due to contamination by excess solvent were identified with the help of Gottlieb et al. [19]; peaks due to short-chain FA and unsaturated FA were identified using Lie Ken Jie and Lam [20]; and Ikeda et al. [21] was useful in differentiating between 1,2- and 1,3-DAG.

Gas Chromatography

Samples were analyzed on a 25 m × 0.25 mm polarizable capillary column lined with a 0.1-μm film of crosslinked 65% phenylmethylsilicone (Quadrex, Woodbridge, CT; part number: 007-65HT-25-0.1F). Polarizable columns separate acylglycerols primarily by carbon number and secondarily by their degree of unsaturation [22, 23]. The column was housed in a Hewlett Packard 5890 (Agilent, Palo Alto, CA) GC equipped with FID and on-column inlet. The inlet pressure for the carrier gas (hydrogen) was set to 15 psi, cool on-column injection was employed and the detector was held at 370 °C. After sample injection, the oven was held at 60 °C for 2 min, then the temperature was increased to 250 °C at 35 °C/min, and finally the temperature was increased to 300 °C at 4 °C/min.

To prepare samples, 50 μL trimethylsilyl-imidazole (TMSI) and 100 μL pyridine was added to approximately 10 mg of synthetic DAG and the mixture was thoroughly mixed using a vortex mixer. The reaction was conducted in a dry nitrogen atmosphere and was complete within 5 min at room temperature [24]. Then, 2 mL of iso-octane was added to the sample vial, the contents were mixed again, and the sample was then allowed to stand for at least 10 min prior to use. The syringe (fused silica needle) was loaded (sandwich technique) in the following order: (1) 1.0 μL of clean solvent, (2) 0.5 μL of air, (3) 0.1 μL from the upper layer of the sample solution, (4) 0.5 μL of air, (5) 1.0 μL of clean solvent and (6) 0.5 μL of air.

Acyl Migration

Conversion between 1,2- and 1,3-DAG occurs mainly by an intramolecular reaction, though it may also occur by intermolecular transesterification. Acyl migration does occur in the solid state, albeit slowly, but it can proceed quite rapidly in the liquid state depending on storage conditions. Acyl migration is promoted by heat, solvents and contact with surfaces such as silica or Florisil™, and is catalyzed by the presence of either acid or base. Over time, an equilibrium mixture of 1,2- and 1,3-DAG is established. This equilibrium favors 1,3-DAG over 1,2-DAG by a ratio of approximately 60:40. The additional stability of the 1,3-DAG is attributed to differences in the nucleophilic character of primary (*sn-1* and *sn-3*) versus secondary (*sn-2*) hydroxyl oxygen atoms [25, 26]. Acyl migration also occurs in MAG, favoring 1(3)- over 2-MAG by approximately 80:20 [27]. The effects of acyl migration were minimized by careful handling and storage of MAG and DAG at −30 °C. To determine the R_f of 1, 2- and 1,3-DAG by TLC, samples of synthetic 1,3-DAG were thermally decomposed in a 60 °C oven overnight.

Results and Discussion

Successful preparation of organic compounds relies on development and execution in three main areas: synthesis, purification and analysis. A weakness in any one of these key areas jeopardizes a project's success. In this paper, 11 diacid and 1 monoacid 1,3-DAG were prepared; the

Table 2 R_f for DAG determined by TLC

		Solvents:	Hexane/EtOAc		$CHCl_3$/acetone
	FA	Ratio: DAG	(7:2) R_f	(3:1) R_f	(96:4) R_f
A	6:0	1,3-	0.32		0.25
	12:0	1,2-	0.22		0.13
B	6:0	1,3-	0.36	0.35	0.29
	16:0	1,2-	0.25	0.25	0.17
C	6:0	1,3-	0.35		0.30
	18:1	1,2-	0.25		0.17
D	12:0	1,3-	0.43	0.40	0.35
	16:0	1,2-			
E	12:0	1,3-	0.41		0.35
	18:1	1,2-	0.29		0.22
F	16:0	1,3-	0.45	0.46	0.38
	18:1	1,2-	0.32	0.34	0.23
G	2:0	1,3-		0.17	0.17
	16:0	1,2-			0.11
H	4:0	1,3-		0.31	0.26
	16:0	1,2-		0.23	0.15
I	8:0	1,3-		0.38	0.33
	16:0	1,2-		0.28	0.20
J	10:0	1,3-		0.39	0.32
	16:0	1,2-		0.29	0.21
K	14:0	1,3-		0.41	0.36
	16:0	1,2-			
L	16:0	1,3-		0.44	0.35
	16:0	1,2-		0.33	0.22

particulars for each compound are summarized in Table 1. This work specifically describes the preparation of racemic 1,3-DAG; substituting enantiopure solketal for racemic solketal would yield enantiopure 1,3-DAG. Yields provided in Table 1 are for the most part approximate and may appear low since we did not try to maximize this parameter, rather, our focus was to obtain sufficient material for further testing. The products were, therefore, usually separated by a series of recrystallizations, often followed by flash chromatography.

Enzymatic Synthesis

This procedure features the creative use of a vinyl ester ⟨9⟩ to minimize the reverse reaction. After esterification, any vinyl alcohol produced instantaneously reconfigures to acetaldehyde ⟨11⟩ (enol → aldehyde)—the more stable tautomer—thereby limiting the reverse reaction (Fig. 1, reaction D). Our original intent was to synthesize diacid 1,3-DAG enzymatically by introducing two different FAVE in sequence. TLC monitoring of some preliminary reactions, however, indicated that the reaction produced 1,3-DAG without appreciable accumulation of 1(3)-MAG intermediate. It appears that 1(3)-MAG was immediately consumed by subsequent reaction with FAVE (i.e., MAG + FAVE → DAG); or with MAG (i.e., MAG + MAG → DAG + glycerol) [28]. DAG was the favored product for one or both of the following reasons: successive products were increasingly soluble in the reaction solvent (solubility: glycerol ≪ MAG < DAG < TAG); or the enzymatic mechanism favors DAG production. While unsuitable for producing diacid 1,3-DAG, the regioselective enzymatic reaction using *Candida antarctica* lipase B was employed to produce the monoacid 1,3-DAG ⟨10⟩ 16:0-OH-16:0 (L) [13]. Enzymatic catalysis is also an effective means for producing MAG from FA that are susceptible to thermal degradation (i.e., PUFA). However, if MAG is the intended product then it is necessary to either use protected glycerol to prevent DAG formation or to follow a more specific procedure [28].

Chemical Synthesis

Diacid 1,3-DAG were produced in two stages, the first FA ⟨2⟩ was used to form a 1(3)-MAG intermediate ⟨4⟩ (Fig. 1, reaction A) and the second FA adduct (⟨5⟩ or ⟨7⟩) was used

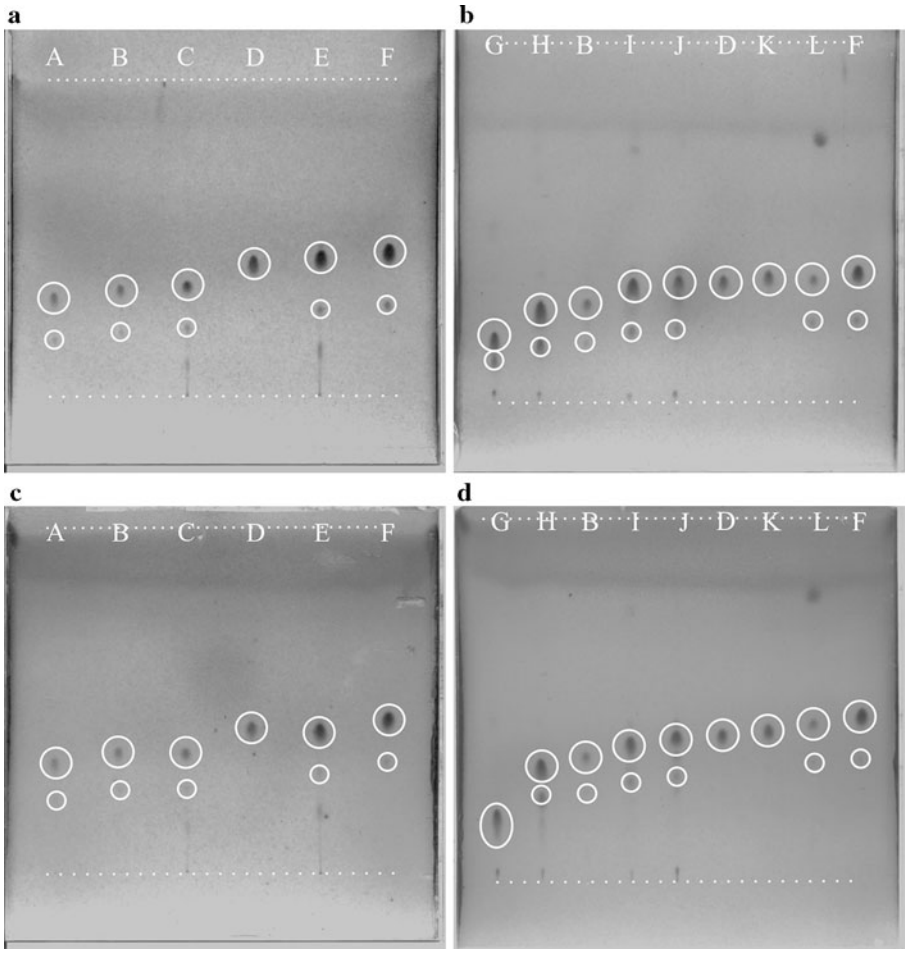

Fig. 2 TLC plates of thermally decomposed DAG: **a**, **b** are boric acid treated plates developed in chloroform/acetone (96:4); **c**, **d** are untreated plates developed in hexane/ethyl acetate: **c** 7:2 and **d** 3:1 (by volume). 1,3-DAG are in *large circles*, 1,2-DAG are in *small circles*, *dotted lines* indicate origin and solvent front maximum. Synthetic 1,3-DAG thermally decomposed to give 1,2- and 1,3-DAG were: A 6:0-OH-12:0, B 6:0-OH-16:0, C 6:0-OH-18:1, D 12:0-OH-16:0, E 12:0-OH-18:1 and F 16:0-OH-18:1, G 2:0-OH-16:0, H 4:0-OH-16:0, B 6:0-OH-16:0, I 8:0-OH-16:0, J 10:0-OH-16:0, D 12:0-OH-16:0, K 14:0-OH-16:0, L 16:0-OH-16:0 and F 16:0-OH-18:1

to form the final product ⟨6⟩ (Fig. 1, reaction B). The progress of the first reaction ⟨i⟩ was monitored by viewing the amount of water collected in the Dean–Stark trap. The MAG intermediate was typically made with the higher-melting FA to facilitate purification by recrystallization.

To produce free 1(3)-MAG ⟨4⟩, the solketal derivative ⟨3⟩ must be deprotected by an acetonide cleavage reaction ⟨ii⟩. Historically this required the use of methoxyethanol, however, due to its toxicity the use of this solvent has been restricted by the Canadian government. Fortunately, a suitable acetonide cleavage procedure involving the use of Amberlyst™ catalyst in ethanol was found [11]. This procedure, involving a 3-h reflux in ethanol, was most appropriate for the production of monopalmitin and was adapted for the production of other MAG. For acetonide cleavage of unsaturates and short-chain containing MAG or to reduce acyl migration, lower reaction temperatures and longer reaction times can be employed [29, 30].

1(3)-MAG were purified by at least one recrystallization and the overall yield for both reactions including recrystallization was ~60%. It was challenging to isolate 1(3)-MAG above 70% purity by recrystallization because these compounds and their monoacid 1,3-DAG counterparts have similar melting points (e.g., 16:0-OH-OH: T_m = 77 °C [31], whereas 16:0-OH-16:0: T_m = 72.0) [32] and their binary mixtures form solid solutions [33].

Diacid 1,3-DAG were synthesized by reaction scheme B or C (Fig. 1) depending on the availability of FA adduct

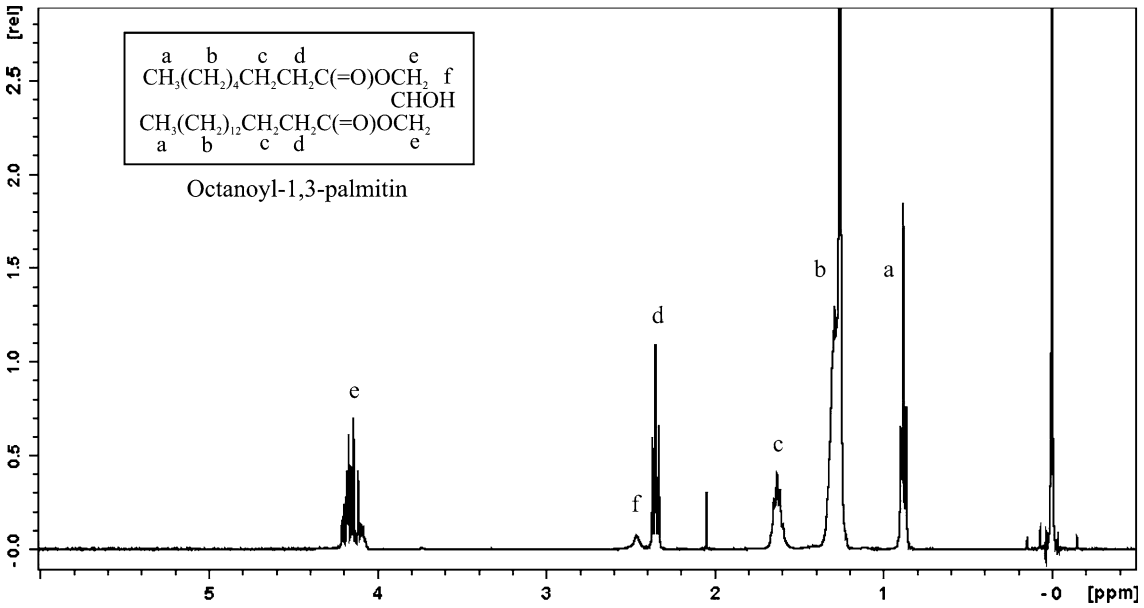

Fig. 3 Nuclear magnetic resonance of octanoyl-1,3-palmitin (8:0-OH-16:0). Peak assignments are provided in the *inset*

Table 3 Chemical shifts determined by proton NMR relative to TMS standard

	Compound	δ_H ($CDCl_3$)
A	6:0-OH-12:0	0.89 (6H, q), 1.25 (20H, m), 1.64 (4H, t), 2.45 (4H, t) and 4.15 (5H, m)
B	6:0-OH-16:0	0.83 (6H, t), 1.29 (28H, d), 1.62 (4H, t), 2.33 (4H, t), 3.73 (2H, m) and 4.09 (5H, m)
C	6:0-OH-18:1	0.82 (6H, m), 1.19 (24H, m), 1.52 (4H, m), 1.90 (4H,m), 2.25 (4H, t), 4.02 (5H, m) and 5.10 (2H, m)
D	12:0-OH-16:0	0.84 (6H, t), 1.22 (40H, d), 1.59 (4H, t), 2.31 (4H, t) and 4.13 (5H, m)
E	12:0-OH-18:1	0.85 (6H, t), 1.25 (36H, t), 1.60 (4H, t), 2.00 (4H, m), 2.32 (4H, t), 4.13 (5H, m) and 5.37 (2H, t)
F	16:0-OH-18:1	0.85 (6H, t), 1.23 (44H, m), 1.69 (4H, m), 2.07 (4H, m), 2.32 (4H, t), 4.20 (5H, m) and 5.35 (2H, m)
G	2:0-OH-16:0	0.88 (3H, t), 1.26 (24H, m), 1.64 (2H, m), 2.31 (3H, s), 2.51 (2H, t), 3.74 (2H, m) and 4.25 (5H, m)
H	4:0-OH-16:0	0.86 (3H, t), 1.01 (3H, t), 1.31 (24H, m), 1.66 (4H, q), 2.44 (4H, m), 3.73 (2H, m) and 4.19 (5H, m)
I	8:0-OH-16:0	0.88 (6H, t), 1.31 (32H, m), 1.63 (4H, q), 2.33 (4H, q), 2.47 (1H, s) and 4.18 (5H, m)
J	10:0-OH-16:0	0.88 (6H, t), 1,30 (36H, d), 1.63 (4H, t), 2.35 (4H, t) and 4.16 (5H, m)
K	14:0-OH-16:0	0.83 (6H, t), 1.29 (44H, m), 1.62 (4H, m), 2.33 (4H, t) and 4.09 (5H, m)
L	16:0-OH-16:0	0.83 (6H, t), 1.29 (48H, m), 1.62 (4H, m), 2.33 (4H, t) and 4.09 (5H, m)

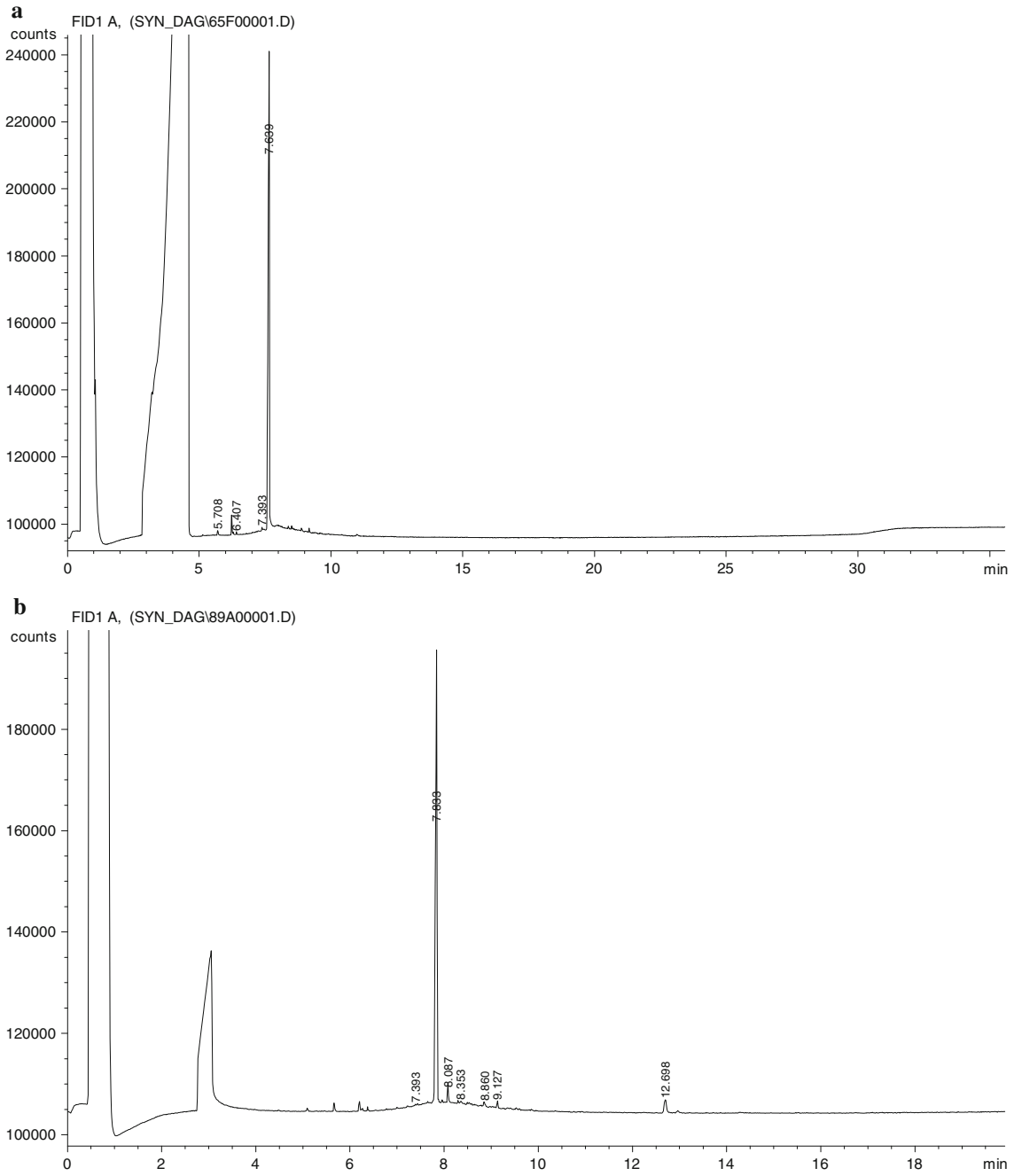

Fig. 4 Gas chromatograms for **a** hexanoyl-1,3-laurin (6:0-OH-12:0) and **b** butyroyl-1,3-palmitin (4:0-OH-16:0). Peaks prior to 5 min are solvent and derivatizing reagent

(acid chloride ⟨5⟩ or acid anhydride ⟨7⟩). The exact scheme used for each compound and the resulting purity of that product is listed in Table 1. For example, 4:0-OH-16:0 (H) ⟨6⟩ was produced via reaction scheme B involving reaction of 1(3)-monopalmitin ⟨4⟩ with butyroyl chloride ⟨5⟩. Raw product from this reaction was separated by flash chromatography for a 24.0% yield of product that was 92.94% pure (by GC). Similarly, 2:0-OH-16:0 (G) ⟨6⟩ was produced via scheme C involving reaction of 1(3)-monopalmitin ⟨4⟩ with acetic anhydride ⟨7⟩. The raw product was separated by flash chromatography for a 21.5% yield of product that was 84.97% pure (by GC).

Purification

Recrystallization, flash chromatography and recrystallization followed by flash chromatography were all employed for post-synthesis isolation of 1,3-DAG. Methods used for

Table 4 GC retention times for 1,3-diacylglycerols

	Compound	RT (min)
A	6:0-OH-12:0	7.64
B	6:0-OH-16:0	8.68
C	6:0-OH-18:1	9.45
D	12:0-OH-16:0	10.55
E	12:0-OH-18:1	11.90
F	16:0-OH-18:1	14.83
G	2:0-OH-16:0	7.50
H	4:0-OH-16:0	7.83
I	8:0-OH-16:0	8.89
J	10:0-OH-16:0	9.60
K	14:0-OH-16:0	11.83
L	16:0-OH-16:0	13.02

final purification of synthetic MAG and DAG are listed in Table 1. Flash chromatography was most effective for isolating low-melting 1,3-DAG ($T_m < 50$ °C) while recrystallization was favored for isolating high-melting 1,3-DAG ($T_m > 50$ °C) and 1(3)-MAG intermediates. Two of the three 1,3-DAG isolated via recrystallization were >99% pure (as determined by GC). On the other hand, of the nine low-melting compounds isolated by flash chromatography, only one was isolated at >99% purity; nevertheless, five were isolated at better than 90% (by GC). One apparent shortcoming of flash chromatography was the lack of compounds obtained with >99% purity (Table 1) (discussed in more detail below).

Three of the 1,3-DAG listed in Table 1 were isolated by recrystallization, 12:0-OH-16:0 and 16:0-OH-16:0 were isolated at >99% (by GC) whereas, 14:0-OH-16:0 was difficult to isolate and was quite impure (72.62% by GC). Isolation of 14:0-OH-16:0 by recrystallization was confounded by the presence of numerous byproducts with similar melting points—the melting points for products and probable byproducts (high-melting forms) in decreasing order are: 16:0-OH-OH (77 °C) [31], 16:0-OH-16:0 (72.5 °C)[31] and 14:0-OH-16:0 (63.5–64 °C) [34]. It was also difficult to isolate 14:0-OH-16:0 by flash chromatography since the R_f of 16:0-OH-16:0 ($R_f = 0.41$) and 14:0-OH-16:0 ($R_f = 0.44$) are virtually the same (discussed below).

Fractions obtained by flash chromatography were less discrete than is commonly assumed. In fact, in some cases, it may be impossible to obtain perfect resolution by this method due to co-elution with other components. This is most easily demonstrated by comparing TLC R_f for the various 1,2- and 1,3-DAG eluted in hexane/ethyl acetate (Table 2; Fig. 2). For example, 6:0-OH-16:0 must be separated from potential byproducts (listed in the order of elution with R_f): 16:0-OH-16:0 ($R_f = 0.44$), 6:0-OH-16:0 ($R_f = 0.35$), 16:0-16:0-OH ($R_f = 0.33$) and 6:0/16:0 1,2-DAG ($R_f = 0.25$) for hexane/ethyl acetate (3:1). Obviously the separation of, 6:0-OH-16:0 and 16:0-16:0-OH will be problematic since their R_f differ (ΔR_f) by only 0.02. For good flash chromatographic separation, components within a sample should have ΔR_f of at least 0.10 and preferably more than 0.15 [18]. This same issue affects all diacid 1,3-DAG with one short and one long acyl chain (e.g., 2:0-OH-16:0, 4:0-OH-16:0). One additional factor to consider is the purity of starting materials. The inability to obtain acyl-1,3-oleins at >95.22% purity may be due to lack of purity in the original starting material (oleic acid).

Analysis

TLC was a key technique for monitoring processes and reactions. It was also used to determine the appropriate solvent ratio for flash chromatography. The chemical structure of the prepared compounds was confirmed by NMR. An example, with peak assignments is provided in Fig. 3; peak assignments for all 1,3-DAG prepared are provided in Table 3. The purity of these compounds was determined by GC analysis of TMS derivatives using a polarizable capillary column (Table 1). TMS derivatives were prepared to preserve the positional isomerism of acyl groups on the glycerol backbone (i.e., to prevent acyl migration) and to enhance the volatility of sample components. Examples of GC chromatograms are provided in Fig. 4; retention times for all 1,3-DAG prepared are provided in Table 4.

While TLC with hexane/ethyl acetate was useful for understanding the isolation of 1,3-DAG by flash chromatography, TLC with chloroform/acetone (96:4) on boric acid-treated plates finds widespread use as an analytical method because it separates positional isomers of partial

Table 5 Tailoring syntheses to the purification procedure

Method	Byproduct or impurity			Reaction time (h)	Equivalents of FA
	MAG	1,2-DAG	TAG		
Recrystallization	Minimize	OK	Preferred	≥3	1.1–1.2
Flash chromatography	Preferred	Minimize	OK	≤2	1.0

acylglycerols (i.e., 1,2- from 1,3-DAG and 2- from 1-MAG) with minimal acyl migration [16]. Evidently, for DAG, R_f is determined by both positional isomerism and the length of the shortest acyl chain (Table 2). This is of little consequence in the analysis of DAG from a typical fat or oil since they have such a narrow range of FA. In contrast, the separation of milk fat DAG by boric acid TLC is exceedingly complex since milk fat has long-, medium- and short-chains. Thus, milk fat DAG separate into numerous fractions on the basis of chain-length and positional isomerism [35].

In retrospect, it is evident that the proportion of reactants and reaction times can be adjusted to achieve better results based on the separation technology employed (Table 5). For example, 1,3-DAG are less soluble in solvent than either TAG or 1,2-DAG but more soluble than MAG, therefore, purification by recrystallization will benefit if TAG rather than MAG is the predominant byproduct. Likewise, for purification by flash chromatography, 1,2- and 1,3-DAG have similar R_f and consequently, can be difficult to separate. Therefore, minimizing 1,2-DAG formation (by reducing reaction times) can be beneficial to chromatographic separation. In addition, MAG tend to be retained near the origin, whereas, TAG elute before DAG, thus, MAG would be favored over TAG as byproducts in this situation. All told, this work contains information critical to lab-scale production of synthetic acylglycerols and provides an excellent starting point for optimal production of 1,3-DAG.

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