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Rapid access to structured triacylglycerols acylated with *n*-3 polyunsaturated fatty acids for nutritional applications

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ABSTRACT

In order to better understand the metabolic fate of *n*-3 polyunsaturated fatty acids (PUFAs), an efficient access to symmetrical and unsymmetrical triacylglycerols (TGs), esterified with PUFAs, with known high purity, is required. In this context, we optimized the esterification of a mixture of glycerols protected as dioxane and dioxolane with PUFAs. The kinetics of this reaction depends on various factors, such as the fatty acid chain length and the stereochemistry of the dioxane. Then, one-pot acetal hydrolysis and esterification of hydroxyl groups led to the desired structured TGs without either double bond isomerization or acyl migration (except when symmetrical TGs are acylated with long-chain saturated fatty acids in external positions). PUFAs location on the glycerol backbone was assayed by NMR, HPLC and pancreatic lipase hydrolysis.

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1. Introduction

For several years, there has been a growing body of evidence on the implication of *n*-3 polyunsaturated fatty acids (PUFAs) on cardiovascular,[1] inflammatory,[2] neurodegenerative diseases[3] and prevention of some cancers.[4] In particular, the beneficial effects on human health of α-linolenic acid (18:3 *n*-3, ALA), eicosapentaenoic acid (20:5 *n*-3, EPA) and docosahexaenoic acid (22:6 *n*-3, DHA) have been pointed out. In the diet, these fatty acids are found esterified as triacylglycerols (TGs) that represent the most important source of lipids. In vivo, the assimilation of the fatty acids present in TGs depends on complex physicochemical and enzymatic processes.[5] In particular, the digestion process of lipid nutrients implies lipolytic enzymes, i.e., gastric and pancreatic lipases. The pancreatic lipase regioselectively hydrolyses the fatty esters of TGs present at the external positions of the glycerol backbone (also called, sn-1 and sn-3 positions). The hydrolysis products formed are two free fatty acids and a 2-monoacylglycerol.[6] Moreover, there is an inherent resistance of very long-chain PUFAs to pancreatic lipase hydrolysis. In other words, the enzyme activity towards short and medium chain is favoured. As a result, the lipase specificity both towards the fatty acid position and the chain length,[7] associated with a preferential intestinal absorption of the 2-monoacylglycerol compared with that of the free fatty acids[8] could account for a favoured in vivo bioavailability of the fatty acids esterified at the position 2 of the glycerol skeleton. Besides these physiological data, the exact influence of the fatty acid position on the glycerol backbone on its metabolic fate is still under investigation. Some studies used structured TGs in nutritional studies.[8a] However, they always concerned mixtures of lipids that prevented understanding the precise metabolic fate of the fatty acids. In this context, it should be helpful to have highly pure structured TGs acylated with PUFAs either in position 1 (or 3) or in position 2, for nutritional investigations.

Two main approaches are commonly used to obtain structured TGs; enzymatic[9] and chemical methods.[9a,10,11] Most of the time, symmetrical TGs are obtained with by-products resulting of acyl migration which occurs as soon as the reaction implies mono- or diacylglycerol intermediates. The synthesis of unsymmetrical TGs is less documented and also suffers from acyl migration.[9b,11a,11c] In general such TGs are obtained in low yields, making these procedures not suitable for large-scale syntheses. Moreover, as PUFAs exhibit pronounced susceptibility to autoxidation affecting integrity of the native olefinic system,[12] the number of available procedures for the preparation of TGs acylated with PUFAs is limited.[9a,b,13] In

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fact, to the best of our knowledge only Fraser and Haraldsson groups have synthesized symmetrical and unsymmetrical TGs, esterified with EPA or DHA, on large-scale. Symmetrical and unsymmetrical TGs prepared by Fraser et al. were obtained, respectively, with 2–5 and 5–10% acyl migration by carbon NMR analysis.[9a] Haraldsson only indicated they have not seen acyl migration with unsymmetrical species after crystallization.[9i]

In order to prevent acyl migration, a synthetic method using glycerol protected as dioxane **1** or dioxolane **3** (Fig. 1) has been developed, and applied so far, only to saturated and monounsaturated fatty acids.[10c,d]

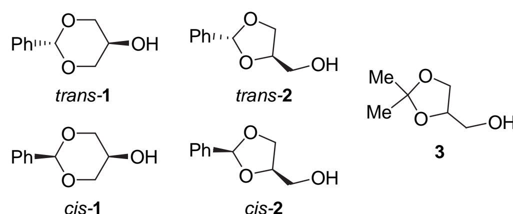


Fig. 1. Five- and six-membered rings obtained from condensation of glycerol with benzaldehyde or acetone.

It is well-known that acetal **1** can easily be obtained by condensation of glycerol and benzaldehyde. But, whatever the reaction conditions, mixtures of 2-phenyl-[1,3]dioxan-5-ol **1** and (2-phenyl-[1,3] dioxolan-4-yl)-methanol **2** are formed after equilibration, in equivalent amounts.[14] Both **1** and **2** also coexist as *cis*/*trans* isomers (Fig. 1).

Usually, *trans*-**1** easily isomerizes, in the presence of acid, into the isomer *cis*-**1**, stabilized thanks to hydrogen bonding between the oxygen atoms and the hydroxyl group in the 1,3-diaxal position.[14g,h] *cis*-**1** can be obtained as the major product after crystallization (Fig. 2).[14f–h,15]

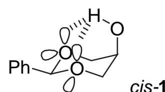


Fig. 2. Stabilizing hydrogen bonding in *cis*-**1**.

If glycerol is condensed with acetone, only the five-membered ring **3** is formed. Thus, *cis*-**1** is used in classical syntheses of symmetrical TGs, while **3** is employed to get unsymmetrical species. The use of a mixture of **1** and **2** to synthesize, simultaneously the precursors of symmetrical and unsymmetrical structured TGs has never been investigated, although such a strategy would lead to both species, in a limited number of reaction steps compared with the number of steps needed in the case of two independent syntheses.

In this paper, we describe, from a mixture of **1** and **2**, a generic approach for the synthesis of new symmetrical and unsymmetrical TGs containing *n*-3 PUFAs (ALA, EPA or DHA). The other positions were esterified either with oleic acid (18:1, OA) as a common fatty acid found in natural oils or with heptadecanoic acid (17:0, HDA), a fatty acid, that is, not present in endogenous lipids of the lymph and that can be used as an internal standard to quantify the intestinal passage of lipids. The different structured TGs chosen to be synthesized in order to use them for in vivo studies in rats are presented in Fig. 3. First, we optimized, on gram-scale, the esterification of **1** with either stearic acid (SA, 16:0) or ALA, and compared the reactivity of the saturated fatty acid to that of the PUFA's. Then, we studied the kinetics of the acylation of the dioxane **1** as a function of its stereochemistry. The best conditions in hands, we prepared a **1**:**2** mixture and proceeded to its acylation with PUFAs. Then, esterified products were transformed into structured TGs in the presence of trifluoroacetic anhydride (TFAA) and OA or HDA.[10d] All products were fully characterized via 1D and/or 2D NMR measurements. TGs' purity was determined by carbon NMR, HPLC and pancreatic lipase hydrolysis.

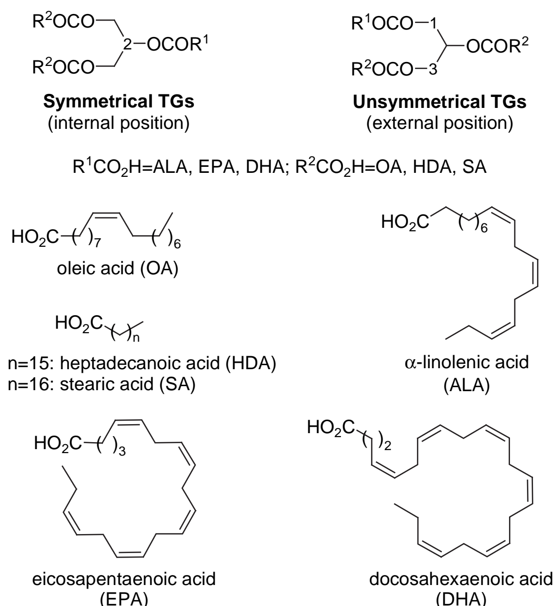


Fig. 3. Structured TGs with PUFAs in internal or external position of the glycerol skeleton.

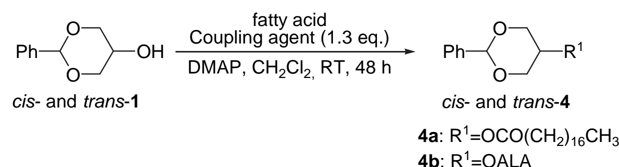
2. Results and discussion

2.1. Optimization of the acylation of protected glycerols

Acylation of **1** with fatty acids is usually described in the presence of coupling agent (DCC or EDCI) and DMAP. However, as amounts of catalyst and reagents vary from one reference to another, it was necessary to determine the appropriate conditions of this reaction in the presence of *n*-3 PUFAs. Thus, we first optimized the esterification of a (1:1) *cis*/*trans* commercial mixture of **1** with either a saturated fatty acid (stearic acid, SA) or a less stable PUFA (α-linolenic acid, ALA). In order to compare the reactivity of both fatty acids, reactions were stopped after 48 h. Acylated products *cis*-**4** and *trans*-**4** were isolated by silica gel chromatography. Some of the conditions used for the esterification and yields of each compound are given in Table 1. Although DMAP can be used in catalytic

Table 1

Esterification of an equimolar *cis*/*trans* mixture of **1**, with either stearic acid (SA) or α-linolenic acid (ALA)



Entry	**1** (equiv)	R^1CO_2H (equiv)	DMAP (equiv)	*cis*-**4**[a] (%)	*trans*-**4**[a] (%)	Total yield (%)
1	1.0	SA (1.2)	0.2	39	23	62[b,d]
2	1.0	SA (1.2)	1.3	39	46	85[b]
3	1.2	SA (1)	1.3	23	53	76[b,d]
4	1.0	SA (1.2)	1.3	37	46	83[c]
5	1.0	ALA (1.2)	0.2	4	4	8[b]
6	1.0	ALA (1.2)	1.3	37	41	78[b]
7	1.0	ALA (1.2)	1.3	39	26	65[c,d]

[a] Yield of isolated chemically pure stereoisomer.
[b] Coupling agent: DCC.
[c] Coupling agent: EDCI.
[d] Recovery of *cis*-**1**; SA: stearic acid; ALA: α-linolenic acid.

amounts with SA, it has to be introduced in slight excess to get higher yields especially with ALA (Table 1, entries 1, 2, 5 and 6). Higher amounts of DMAP permit to enhance the reaction kinetics and to obtain **4b** in better yields (Table 1, entry 6). These results indicate that ALA reacts more slowly than the saturated fatty acid.

Furthermore, a slight excess of fatty acid leads to better yields than an excess of alcohol and avoids the presence of unconsumed *cis*-**1**, difficult to remove during the purification step (Table 1, entries 2 and 3).

To facilitate the purification, DCC was replaced by EDCI.[9a] With SA, the choice of the coupling agent has no influence on the reaction yield (Table 1, entries 2 and 4), whereas in our hands, in the presence of ALA, DCC leads to better results (Table 1, entries 6 and 7). This study also shows that ratios of acylated products *cis*-**4** and *trans*-**4** depend on the experimental conditions. Indeed, although the esterification was carried out from a (1:1) mixture of *cis*/*trans*-**1**, stereoisomers of **4** are not obtained in equivalent amount. Most of the time, *trans*-**4** is obtained as the main product (Table 1, entries 2–4 and 6) except in the presence of catalytic amount of DMAP or when **1** is esterified with ALA in the presence of EDCI (Table 1, entries 1 and 7). These results suggest that *cis* and *trans* stereoisomers of **1** have not the same reactivity towards the carboxylic acid. To check this hypothesis we followed by ^{1}H NMR measurements, the acylation of a (1:0.96) *cis*/*trans* mixture of **1**, with SA and 0.5 equiv of DMAP (Fig. 4). In such conditions, the reaction of *trans*-**1** is complete in 20 min and, according to the integration values, no isomerization of *trans*-**1** into *cis*-**1** occurs. Regarding the *cis* stereoisomer, only half of *cis*-**1** has reacted within 20 min. The esterification of this species is almost complete in 2 h 50 min (96% conversion) and fully complete after 24 h.

As mentioned previously, *cis*-**1** is stabilized thanks to hydrogen bonds between the hydroxyl group and the oxygen atoms of the ring. Moreover, as detailed below, *trans*-**4** adopts a chair conformation while the *cis* stereoisomer adopts a less stable twist one. Both the hydrogen bonds stabilization in *cis*-**1** and the formation of the less stable *cis*-**4** product may disfavour the formation of *cis*-**4**. Nevertheless, when only 0.2 equiv of DMAP are used, we observe an inversion of the stereoisomer ratio, in favour of *cis*-**4a**. In such conditions, the esterification is slowed down and partial isomerization of *trans*-**1** into *cis*-**1** becomes possible before the esterification is achieved.[14g,h] As a result, at the end of the reaction, more *cis*-**4a** than *trans*-**4a** is recovered.

2.2. Efficient access to structured TGs esterified with various PUFAs

After having optimized the esterification of **1** with ALA, we prepared, in 80% yield, a (42:58) **1**:**2** mixture from glycerol and

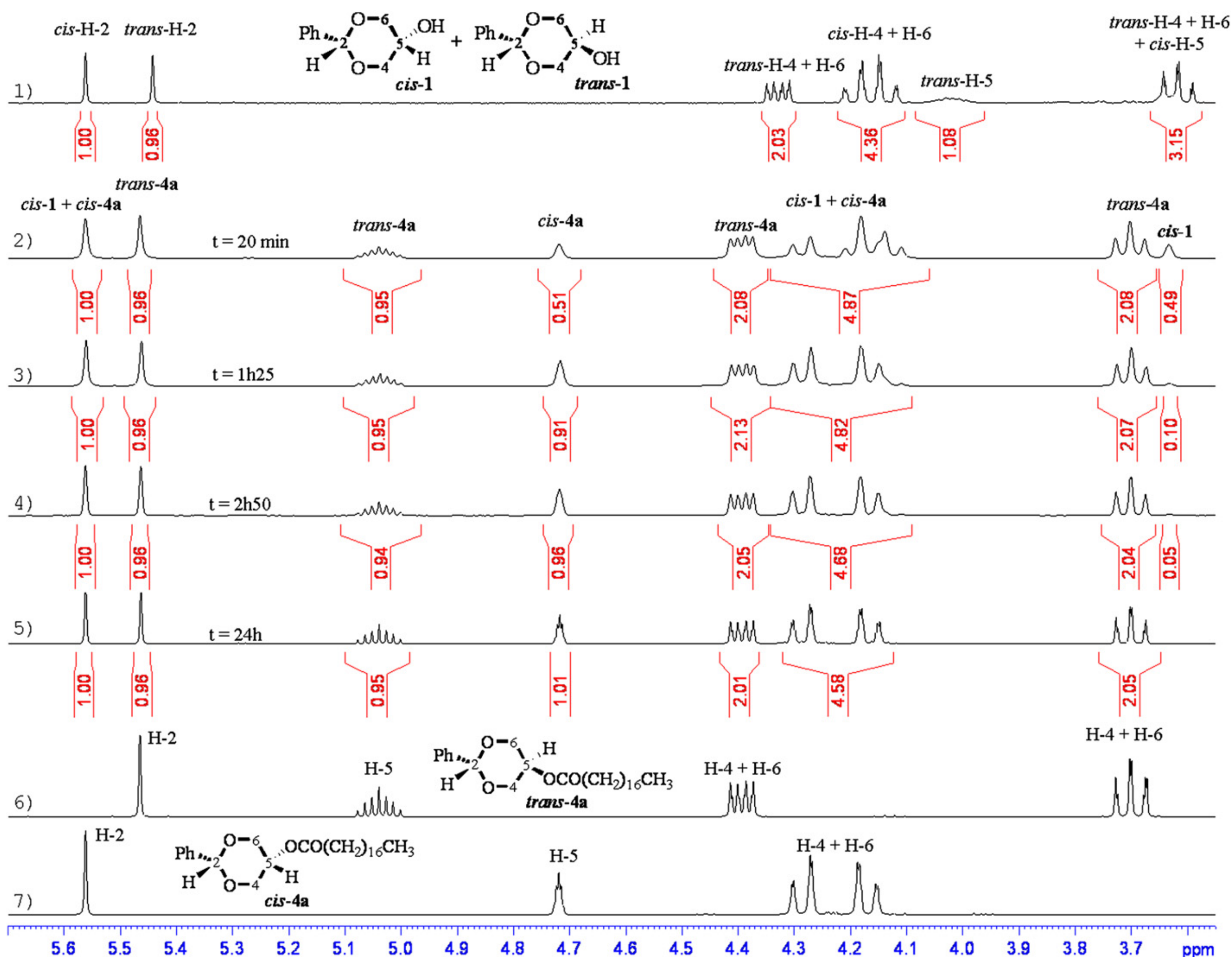
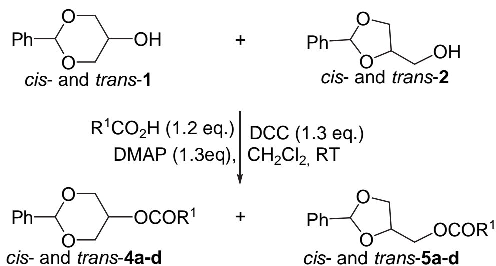
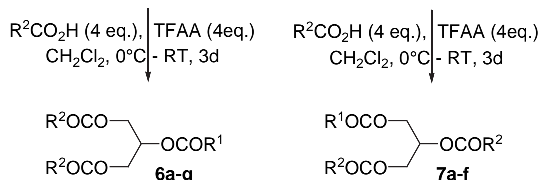


Fig. 4. ^{1}H NMR spectra, in $CDCl_3$ at 400 MHz, as a function of time of a *cis*/*trans* mixture of **1** in the absence and in the presence of stearic acid (1.3 equiv), DCC (1.3 equiv) and DMAP (0.5 equiv) at 25 °C. Mixture of *cis*/*trans*-**1** (1). Reaction after 20 min (2), 1 h 25 min (3), 2 h 50 min (4) and 24 h (5). Pure products *trans*-**4a** (6) and *cis*-**4a** (7).

benzaldehyde[14a] (Scheme 1). To verify if a difference of reactivity existed between the stereoisomers of dioxolane **2**, the esterification of this **1**:**2** mixture was studied by ^{1}H NMR with SA. In contrast with the stereoisomers of **1**, the stereoisomers of **2** have the same reaction kinetics and are esterified as fast as *trans*-**1** (see Supplementary data).



a: R^1CO_2H = SA; b: R^1CO_2H = ALA; c: R^1CO_2H = EPA; d: R^1CO_2H = DHA



a: R^1CO_2H = ALA, R^2CO_2H = HDA; b: R^1CO_2H = ALA, R^2CO_2H = OA
c: R^1CO_2H = EPA, R^2CO_2H = HDA; d : R^1CO_2H = EPA, R^2CO_2H = OA
e: R^1CO_2H = DHA, R^2CO_2H = HDA; f : R^1CO_2H = DHA, R^2CO_2H = OA
g: R^1CO_2H = SA, R^2CO_2H = HA

ALA: α-linolenic acid; EPA: eicosapentaenoic acid
DHA: docosahexaenoic acid; HA: heptanoic acid
HDA: heptadecanoic acid; OA: oleic acid; SA: stearic acid

Scheme 1. Acylation of the **1**:**2** mixture with stearic acid or polyunsaturated fatty acids and access to structured TGs.

Then, in the optimized conditions determined previously (DCC (1.3 equiv), DMAP (1.3 equiv) and fatty acid (1.2 equiv)), the **1**:**2** mixture was successfully esterified with SA and PUFAs of the *n*-3 series forming **4** and **5**. As mentioned before, reactions were stopped after 48 h, in order to compare the reactivity of each PUFA. At this stage, to check if each stereoisomers of **4** and **5** reacted differently or not at the time of the last reaction step, we decided to purify and isolate all these stereoisomers by chromatography on silica gel. Results are summarized in Table 2. Products **4** and **5** were isolated in excellent yields when the acylation was made with the saturated fatty acid or with ALA (Table 2, entries 1 and 2) but the yield decreases with the chain length and the unsaturation degree of the fatty acid (Table 2, entries 1– 4), as already reported in the literature from 3.[9b] The lowest yield, obtained with DHA, might come from either its higher instability to oxidation or its structure.

Table 2
Acylation, with fatty acids, of the mixture of alcohols **1** and **2**

Entry	R^1CO_2H	*cis*-**4**[a] (%)	*trans*-**4**[a] (%)	*cis*-**5**[a] (%)	*trans*-**5**[a] (%)	Reaction yield (%)
1	SA	25	14	33	27	99[b]
2	ALA	30	14	25	27	96[b]
3	EPA	8	17	35	22	82[b]
4	DHA	10	6	23	17	56[b]
5	DHA	21	15	32	28	96[c]

SA: stearic acid; ALA: α-linolenic acid; EPA: eicosapentaenoic acid; DHA: docosahexaenoic acid.
[a] Yield of isolated chemically pure stereoisomer.
[b] Reaction time: 48 h.
[c] Reaction time: 96 h.

To answer this question, the reaction time of the acylation in the presence of DHA was increased. After 4 days, the yield reached 96% (Table 2, entry 5). This result proves that PUFAs are stable enough under these experimental conditions and also indicates that the structure of the PUFA has an influence on the kinetic of the reaction. The more the PUFA is long and unsaturated, the more the reaction is slow, as if the coiled conformation of the PUFA, due to the *cis* configuration of the double bonds, was responsible for steric hindrance during the acylation.

Each stereoisomer of products **4** and **5** has been fully characterized by ^{1}H and ^{13}C NMR. The assignment of *cis* and *trans* stereoisomers as well as the conformations of the six-member rings were deduced from both proton and 2D NOESY NMR experiments (see Supplementary data). For instance in the case of the dioxane *trans*-**4b** we observed a correlation between the benzylic proton H-2 and the axial protons H-4 and H-6, whereas no correlation was observed between H-2 and the equatorial protons. The configuration of *cis*-**4b** is confirmed by the observed correlation between the proton H-2 and the proton H-5. Likewise for the dioxolane **5b**, a correlation is observed in the *cis* isomer spectrum between protons H-2 and H-4 but not in the *trans* isomer one. Moreover, according to proton NMR experiment, the ring of *trans*-**4** adopts a chair conformation. This is supported by the coupling constants of hydrogen atoms at C-4 (or C-6) of the ring ($J_{H\text{-}4ax-H\text{-}5}$=10 Hz; $J_{H\text{-}4eq-H\text{-}5}$=5.2 Hz; J_{gem}=11.2 Hz). In contrast, the chair conformation is excluded in the case of *cis*-**4b** by its proton NMR spectrum. Indeed, this spectrum shows a large *gem* coupling (J_{gem}=12.9 Hz) and only small coupling constants (J=1.8 and 1.3 Hz) between H-5 and both H-4 (or H-6).[16] According to Karplus law,[17] these coupling constants indicate that the dihedral angles between H-5 and H-4 (or H-6) are around 60–70° and they are in favour of a distorted conformation. As correlations between H-5 and both geminal protons H-4 (and H-6) are observed the NOESY spectrum, this is probably a twist conformation in which phenyl and ester groups adopt pseudo-equatorial positions.

Then, we carried out the synthesis of the structured TGs shown in Scheme 1, following the procedure developed in the case of **4** acylated with saturated or monounsaturated fatty acids.[10c,d] In order to assess the influence of the stereochemistry of **4** and **5** on the recovery of symmetrical TGs **6** and unsymmetrical TGs **7**, the synthesis was performed with products **4** and **5** present as a mixture of *cis*/*trans* isomers or as pure stereoisomers (Scheme 1, Table 3). In the presence of TFAA and OA (or HDA), TGs **6** and **7** were successfully obtained from **4** and **5**, acylated with PUFAs.

Table 3
TGs recovery from **4** and **5** used as pure stereoisomers or as a mixture of *cis*/*trans* stereoisomers

Entry	Acetal	TG	R^1CO_2H	R^2CO_2H	Yields of TG (%)
1	*cis*-**4b**	**6a**	ALA	HDA	48
2	*trans*-**4b**	**6a**	ALA	HDA	46
3	*cis*-**4b**	**6b**	ALA	OA	35
4	*trans*-**4b**	**6b**	ALA	OA	38
5	**4c**[a]	**6c**	EPA	HDA	46
6	**4c**[a]	**6d**	EPA	OA	44
7	**4d**[a]	**6e**	DHA	HDA	46
8	**4d**[a]	**6f**	DHA	OA	47
9	*trans*-**4a**	**6g**	SA	HA	71
10	*trans*-**5b**	**7a**	ALA	HDA	63
11	*cis*-**5b**	**7a**	ALA	HDA	60
12	**5b**[a]	**7a**	ALA	HDA	68
13	**5b**[a]	**7b**	ALA	OA	70
14	**5c**[a]	**7c**	EPA	HDA	44
15	**5d**[a]	**7e**	DHA	HDA	63
16	**5d**[a]	**7f**	DHA	OA	60

[a] *cis*/*trans* mixture of stereoisomers; SA: stearic acid; ALA: α-linolenic acid; EPA: eicosapentaenoic acid; HA: Heptanoic acid; HDA: Heptadecanoic acid; DHA: docosahexaenoic acid; OA: oleic acid.

Symmetrical TGs **6** were obtained with moderate to good yields. With SA (Table 3, entry 9) the yield is in agreement with those reported in the literature for saturated species.[10d] With PUFAs, yields are lower probably due to possible fatty acid non linear conformation (Table 3, entries 1–8). For ALA, yields are similar whatever the stereochemistry of the starting compound **4b** (Table 3, entries 1–4). Therefore, other symmetrical TGs, with longer fatty acid chains, were synthesized from a *cis/trans* mixture of **4c** or **4d**. Based on these results, it is evident that the isolated yields do not depend on the identity of the PUFA (Table 3, entries 1, 2, 5, 7 and 3, 4, 6, 8).

From **5**, unsymmetrical TGs acylated with PUFAs were synthesized for the first time with very good yields. As for **4**, the stereochemistry of the starting material does not affect the reaction (Table 3, entries 10 and 11).

Knowing the reactivity of each stereoisomers of **4** and **5**, we attempted the simultaneous synthesis of symmetrical and unsymmetrical TGs **6** and **7** from a mixture of unpurified **4** and **5**. Although the reaction proceeds smoothly the obtained regioisomers **6** and **7** have too similar polarities to be separated, even on impregnated silver nitrate silica gel.[11a,18]

2.3. Acyl migration and enzymatic hydrolysis of structured triacylglycerols

As acyl migration (that depends on many factors including temperature, solvent and presence of traces of acid or base) can be a major problem during the synthesis and purification of TGs, the regiochemical identity of the synthesized species was carefully checked.

According to ^{13}C NMR measurements, no signal corresponding to acyl migration was detected except in the case of the symmetrical product **6e** (see Supplementary data). Indeed, each of the symmetrical TGs contains the expected carbonyl resonances in a ratio 2:1 while the unsymmetrical TGs contain the expected three resonances in a ratio 1:1:1. Carbon NMR spectra of the carbonyl region of structured TGs acylated with ALA, are given as an example in Fig. 5.

Then, symmetrical and unsymmetrical TGs containing ALA were submitted to pancreatic lipase action, a method classically used to quantify the presence of fatty acid esterified at the different positions of the glycerol skeleton.[19] Table 4 presents the ALA distribution determined by the pancreatic lipase method. From symmetrical TGs **6a** and **6b**, 93.6 and 91.8% of ALA was recovered, respectively, in internal position, i.e., as 2-monoglycerides after lipase hydrolysis (Table 4, entries 1 and 2). However, 6.4–8.2% of ALA was measured at the external position since 2-monoglycerides acylated either with OA or HDA were also recovered. From unsymmetrical TGs **7a** and **7b**, 2-monoglycerides were mainly acylated with OA or HDA and only less than 1% of ALA was measured at the internal position (Table 4, entries 3 and 4).

Table 4
Amount of fatty acids in internal position, after enzymatic hydrolysis of structured triacylglycerols acylated with ALA

Entry	TG	ALA in internal position[a] (%)	OA in internal position[a] (%)	HDA in internal position[a] (%)
1	**6a**	93.6	—	6.4
2	**6b**	91.8	8.2	—
3	**7a**	0.7	—	99.3
4	**7b**	0.9	99.1	—

[a] Determined by gas chromatography.

As the detection limit of the NMR experiment is about 5–10%, and as the enzymatic hydrolysis is known to lead to some acyl migration,[20] we also quantified the purity of TGs by Ag-HPLC. In the case of symmetrical TGs, acyl migration products are detected only when glycerol backbone is esterified with HDA. For instance, TG **6a** is obtained with 8.0% of the acyl migration product **7a** (Fig. 6b) while **6b**, acylated with OA is obtained in a pure form (Fig. 6e). Thus, the 8.2% of OA in internal position determined after lipase hydrolysis of **6b**, only comes from the pancreatic analysis method, but not from the synthesis. These results are in agreement with those previously reported by Xu et al.[20] In the case of enzymatic TGs synthesis, they showed that long-chain saturated fatty acids were more subject to acyl migration than unsaturated ones.

As shown in Fig. 6c and f, it is noteworthy that no acyl migration occurs during the synthesis of unsymmetrical TGs.

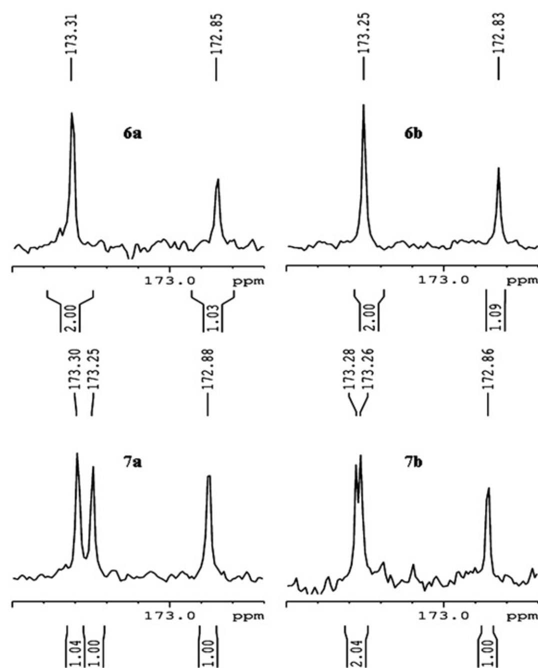


Fig. 5. Estimation of the purity of symmetrical and unsymmetrical TGs acylated with ALA (^{13}C NMR experiments in $CDCl_3$, at 25 °C).

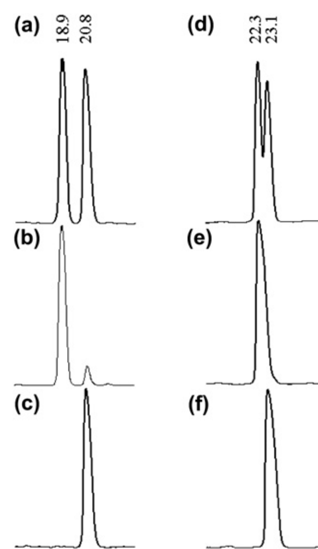


Fig. 6. Ag-HPLC analysis of symmetrical and unsymmetrical TGs acylated with ALA. (a) HPLC chromatogram of a mixture of **6a** and **7a**. (b) HPLC chromatogram of **6a** (c) HPLC chromatogram of **7a**. (d) HPLC chromatogram of a mixture of **6b** and **7b**. (e) HPLC chromatogram of **6b**. (f) HPLC chromatogram of **7b**. Column: Chromspher Lipids® Varian, 250×4.6 mm. Mobile phase: 99.25/0.75 hexane/acetonitrile. Flow rate: 1 mL/min. UV detection at 206 nm.

3. Conclusions

In summary, we have demonstrated that the esterification of a (**1**:**2**) mixture was possible with excellent yields, even with long-chain PUFAs. The resulting esters were transformed successfully into structured TGs with high purity according to NMR, HPLC and enzyme hydrolysis experiments. No sign of isomerization of unstable non-conjugated double bonds into conjugated ones has been observed. Besides, acyl migration in symmetrical TGs is observed only when long-chain saturated fatty acids are present at the external positions of the glycerol backbone. No acyl migration was observed when OA is used or in the case of unsymmetrical TGs. Because of the high purity of these TGs, they are currently being used for in vivo studies in rats in order to get specific data concerning PUFAs metabolism.

4. Experimental section

4.1. General

All reactions were carried out under nitrogen atmosphere using dried glassware and syringe/septa techniques. All commercial reagents were purchased from Aldrich and used without further purification. Dichloromethane, ethyl acetate and hexane with spectrophotometric grade were purchased from Scharlau. Dichloromethane was freshly distilled from CaH_2 before use. The mixture of **1** and **2** was prepared according to a procedure described in literature.[14a] ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Ultrashield Avance 400 MHz spectrometer. J values are given in hertz. Chemical shifts are reported relative internal tetramethylsilane (δ 0.00 ppm) or $CDCl_3$ (δ 7.26 ppm) for ^{1}H and $CDCl_3$ (δ 77.0 ppm) for ^{13}C. The following notation is used for the ^{1}H NMR spectral splitting patterns: singlet (s), doublet (d), triplet (t), multiplet (m), doublet of doublet (dd), triplet of triplet (tt). Infrared spectra were determined on a Nicolet iS10 FT-IR spectrometer. Melting points were measured using a Kofler Heizbank melting point bench (model 7841). High Resolution Liquid Secondary Ion Mass Spectrometry was carried out on a Fisons Autospec Mass Spectrometer as an ABEqQ Configuration. Elemental analyses were performed at the CNRS Center of Chemical Analysis in Vernaison (France). The intramolecular fatty acid distribution in TGs was determined through lipase-catalysed hydrolysis experiments. The resulting sn-2 monoacylglycerols and free fatty acids were separated by TLC. Respective fractions were transmethylated in the presence of boron trifluoride/methanol. Fatty acid methyl esters of sn-2 monoacylglycerols and of free fatty acids were subjected to gas chromatography (GC) on a BPX 70 capillary column (60-m long, 0.25-μm film, 0.25-mm *i.d.*, SGE, hydrogen as carrier gas, split ratio of 1:80). The gas chromatography system consisted in a gas chromatograph (Hewlett–Packard, HP 4890) provided with a flame ionization detector temperature maintained at 250 °C. The injector temperature was at 250 °C. The column temperature was programmed from 150 °C to 200 °C (1.5 °C/min) held for 50 min, from 200 °C to 250 °C (20 °C/min), held for 20 min. Data was collected and integrated by a Chromjet SP 4400 integration system (Spectra-Physics). Fatty acids of known composition from Sigma were used as standards for column calibration. Variation in surface area determination between injections was less than 2%. Ag-HPLC analyses were carried out on Perkin–Elmer series 200 apparatus with 250×4.6 mm ChromSpher Lipids® column of Varian.

4.2. General procedure for the synthesis of pure esters 4 and 5

To a mixture of DCC (745 mg, 3.6 mmol), DMAP (440 mg, 3.6 mmol) and fatty acid (3.35 mmol) in freshly distilled CH_2Cl_2 (20 mL) was added, under nitrogen atmosphere, the mixture of **1** and **2** (500 mg, 2.75 mmol). The resulting solution was stirred for 48 h or 96 h at room temperature. The solvent was evaporated in vacuo to give a crude mixture of four stereoisomers, which was purified by silica gel column chromatography (10% AcOEt/hexane) to give pure *cis* and *trans* stereoisomers of **4** and **5**.

Compounds **4b** and **5b** are given as examples. Synthesis and characterization data of all esters **4** and **5** are described in the Supplementary data.

*4.2.1. (9Z,12Z,15Z)-Octadeca-9,12,15-trienoic acid 2-phenyl-[1,3]dioxan-5-yl ester **4b** and (9Z,12Z,15Z)-octadeca-9,12,15-trienoic acid 2-phenyl-[1,3]dioxolan-4-ylmethyl ester **5b**.* Stereoisomers of **4b** and **5b** were prepared from α-linolenic acid (932 mg) in 96% yield (1.163 g, 2.64 mmol).

*4.2.2. Compound cis-**4b**.* Yield (363 mg, 30%) as colourless oil; R_f (10% AcOEt/hexane) 0.18; ν_{max} (KBr) 3095, 3070, 3009, 2960, 2927, 2853, 1732, 1641, 1525, 1453, 1394, 1238, 1144 and 1084 cm^{-1}; δ_H (400 MHz; $CDCl_3$; Me_4Si) 7.52–7.49 (2H, m), 7.38–7.36 (3H, m), 5.56 (1H, s), 5.38–5.33 (6H, m), 4.72 (1H, tt, J=1.8 and 1.3), 4.29 (2H, dd, J=12.9 and 1.3), 4.17 (2H, dd, J=12.9 and 1.8), 2.82–2.79 (4H, m), 2.43 (2H, t, J=7.6), 2.09–2.03 (4H, m), 1.70–1.60 (2H, m), 1.34–1.32 (8H, m) and 0.97 (3H, t, J=7.6); δ_C (100 MHz, $CDCl_3$, Me_4Si) 173.9, 137.9, 132.0, 130.3, 129.1, 128.3, 127.7, 127.2, 126.1, 101.3, 69.2, 65.8, 34.4, 29.6, 29.2, 29.13, 29.10, 27.2, 25.6, 25.5, 24.94, 20.7 and 14.3; m/z (ESI$^+$) 463.5 ([M+Na]$^+$, 100%); HRMS (ESI$^+$) calcd for $C_{28}H_{40}O_4Na$ 463.2824 found 463.2803.

*4.2.3. Compound trans-**4b**.* Yield (169 mg, 14%) as colourless oil; R_f (10% AcOEt/hexane) 0.45; ν_{max} (KBr) 3096, 3068, 3010, 2958, 2927, 2854, 1738, 1642, 1523, 1450, 1392, 1235, 1144 and 1083 cm^{-1}; δ_H (400 MHz; $CDCl_3$; Me_4Si) 7.49–7.47 (2H, m), 7.38–7.34 (3H, m), 5.46 (1H, s), 5.41–5.29 (6H, m), 5.03 (1H, tt, J=10.0 and 5.2), 4.38 (2H, dd, J=11.2 and 5.2), 3.69 (2H, dd, J=11.2 and 10.0), 2.82–2.79 (4H, m), 2.30 (2H, t, J=7.6), 2.09–2.03 (4H, m), 1.70–1.60 (2H, m), 1.34–1.32 (8H m) and 0.97 (3H, t, J=7.6); δ_C (100 MHz, $CDCl_3$, Me_4Si) 172.7, 137.3, 132.0, 130.2, 129.2, 128.4, 128.3, 128.2, 127.8, 127.1, 126.1, 104.4, 68.6, 62.6, 34.1, 29.6, 29.13, 29.08, 29.05, 27.2, 25.6, 25.5, 24.8, 20.6 and 14.3; m/z (ESI$^+$) 463.5 ([M+Na]$^+$, 100%); HRMS (ESI$^+$) calcd for $C_{28}H_{40}O_4Na$ 463.2824 found 463.2805.

*4.2.4. Compound cis-**5b**.* Yield (304 mg, 25%) as colourless oil; R_f (10% AcOEt/hexane) 0.25; ν_{max} (KBr) 3108, 3093, 3065, 3009, 2960, 2926, 2850, 1728, 1644, 1470, 1394, 1212, 1094 and 1065 cm^{-1}; δ_H (400 MHz; $CDCl_3$; Me_4Si) 7.64–7.61 (2H, m), 7.39–7.37 (3H, m), 5.82 (1H, s), 5.43–5.28 (6H, m), 4.47–4.43 (1H, m), 4.24–4.22 (2H, m), 4.12 (1H, dd, J=8.4 and 7.2), 3.95 (1H, dd, J=8.4 and 5.2), 2.82–2.79 (4H, m), 2.33 (2H, t, J=7.2), 2.09–2.03 (4H, m), 1.66–1.60 (2H, m), 1.40–1.27 (8H, m) and 0.97 (3H, t, J=7.6); δ_C (100 MHz, $CDCl_3$, Me_4Si) 173.6, 137.0, 132.0, 130.2, 129.5, 128.4, 128.3, 128.2, 127.7, 127.1, 126.7, 104.7, 74.2, 67.5, 64.3, 34.1, 29.6, 29.13, 29.07, 27.2, 25.6, 25.5, 24.9, 20.5 and 14.3; m/z (ESI$^+$) 463.5 ([M+Na]$^+$, 100%); HRMS (ESI$^+$) calcd for $C_{28}H_{40}O_4Na$ 463.2824 found 463.2816.

*4.2.5. Compound trans-**5b**.* Yield (327 mg, 27%) as colourless oil; R_f (10% AcOEt/hexane) 0.37; ν_{max} (KBr) 3112, 3091, 3066, 3010, 2958, 2925, 2853, 1735, 1641, 1470, 1393, 1212, 1096 and 1066 cm^{-1}; δ_H (400 MHz; $CDCl_3$; Me_4Si) 7.48–7.46 (2H, m), 7.39–7.36 (3H, m), 5.95 (1H, s), 5.43–5.28 (6H, m), 4.52–4.60 (1H, m), 4.29–4.20 (3H, m), 3.79 (1H, dd, J=8.4 and 6.6), 2.82–2.79 (4H, m), 2.33 (2H, t, J=7.2), 2.09–2.03 (4H, m), 1.66–1.60 (2H, m), 1.40–1.27 (8H, m) and 0.97 (3H, t, J=7.6); δ_C (100 MHz, $CDCl_3$, Me_4Si) 173.6, 137,5, 132.0, 130.2, 129.3, 128.4, 128.3, 128.2, 127.7, 127.1, 126.4, 103.9, 73.9, 67.3, 63.9, 34.1, 29.6, 29.2, 29.1, 27.2, 25.6, 25.5, 24.9, 20.5 and 14.3; m/z

(ESI$^+$) 463.5 ([M+Na]$^+$, 100%); HRMS (ESI$^+$) calcd for $C_{28}H_{40}O_4Na$ 463.2824 found 463.2820.

4.3. General procedure to access to structured triacylglycerols

To a mixture of **4** or **5** (1 equiv) and fatty acid (4 equiv) in anhydrous CH_2Cl_2 (3 mL/mmol) at 0 °C was added slowly TFAA (4 equiv). At the end of the addition, the resulting brown solution was stirred at room temperature for 3 days. The solvent was evaporated in vacuo and the crude residue purified by silica gel chromatography (5% AcOEt/hexane) to give pure triacylglycerol **6** or **7**.

All compounds **6** and **7** were prepared according to this procedure. Compounds **6a** and **7a** are given as examples. Details concerning the synthesis and the characterization data of other structured triacylglycerols are given in the Supplementary data.

*4.3.1. Octadeca-9,12,15-trienoic acid 2-heptadecanoyloxy-1-heptadecanoyloxymethyl-ethyl ester **6a**.* Compound **6a** was prepared from **4b** (306 mg, 0.695 mmol) and heptadecanoic acid (712 mg, 2.78 mmol) in 48% yield (285 mg, 0.33 mmol) as colourless oil; [Found: C, 76.77; H, 11.47. $C_{55}H_{100}O_6$ requires C, 77.05; H 11.76%]; R_f (20% AcOEt/hexane) 0.78; ν_{max} (KBr) 2921, 2851, 1743, 1466, 1253, 1165, 1098 and 717 cm^{-1}; δ_H (400 MHz; $CDCl_3$; Me_4Si) 5.45–5.26 (7H, m), 4.29 (2H, dd, J=11.9 and 4.3), 4.14 (2H, dd, J=11.9 and 5.9), 2.82–2.79 (4H, m), 2.40–2.25 (6H, m), 2.12–2.01 (4H, m), 1.67–1.55 (6H, m), 1.45–1.15 (60H, m), 0.97 (3H, t, J=7.6) and 0.88 (6H, t, J=7.2); δ_C (100 MHz, $CDCl_3$, Me_4Si) 173.3, 172.8, 131.9, 130.2, 128.3, 128.2, 127.8, 127.1, 68.9, 62.1, 34.2, 34.0, 31.9, 29.71, 29.68, 29.64, 29.62, 29.5, 29.4, 29.3, 29.2, 29.1, 29.0, 27.2, 25.6, 25.5, 24.9, 22.7, 20.6, 14.3 and 14.1.

*4.3.2. Octadeca-9,12,15-trienoic acid 2,3-bis-heptadecanoyloxy-propyl ester **7a**.* Compound **7a** was prepared from **5b** (205 mg, 0.465 mmol) and heptadecanoic acid (477 mg, 1.86 mmol) in 68% yield (272 mg, 0.32 mmol) as colourless oil; R_f (20% AcOEt/hexane) 0.78; ν_{max} (KBr) 2851, 1743, 1466, 1253, 1165, 1098 and 717 cm^{-1}; δ_H (400 MHz; $CDCl_3$; Me_4Si) 5.44–5.23 (7H m), 4.29 (2H, dd, J=11.9 and 4.3), 4.14 (2H, dd, J=11.9 and 6.0), 2.85–2.75 (4H, m), 2.38–2.27 (6H, m), 2.13–2.02 (4H, m), 1.68–1.53 (6H, m), 1.45–1.2 (60H, m), 0.97 (3H, t, J=7.6) and 0.88 (6H, t, J=7.2); δ_C (100 MHz, $CDCl_3$, Me_4Si) 173.3, 173.2, 172.9, 132.0, 130.2, 128.3, 128.2, 127.8, 127.1, 68.9, 62.1, 34.2, 34.1, 34.0, 31.9, 29.72, 29.68, 29.64, 29.61, 29.52, 29.50, 29.4, 29.31, 29.29, 29.2, 29.14, 29.10, 27.2, 25.6, 25.5, 24.93, 24.89, 24.85, 22.7, 20.6, 14.3 and 14.2; m/z (ESI$^+$) 879 ([M+Na]$^+$, 100%); HRMS (ESI$^+$) calcd for $C_{55}H_{100}O_6Na$ 879.7412 found 879.7398.

4.4. Determination of the fatty acid distribution in TGs using lipase hydrolysis

The intramolecular fatty acid distribution in TGs was determined according to a method described with slight modifications.[19] Briefly, lipase-catalyzed hydrolysis experiments were performed in a thermostated bath at 40 °C and pH 8. The reaction mixture consisted of 15 mg of TGs, 1 g of porcine pancreatin suspended in 1 mL of Tris–HCl buffer (1.0 M adjusted to a pH of 8.0), 0.2 mL of aqueous solution of calcium chloride (22%; wt/vol) and 0.5 mL of aqueous solution of sodium deoxycholate (0.1%; wt/vol). The resulting 2-monoacylglycerols and free fatty acids were separated by thin layer chromatography (TLC). TLC was performed with Merck silica gel (60H) spread on 20×20 cm glass plates, 0.35 mm thick, activated at 110 °C for 1 h hexane/diethyl ether/formic acid (70/30/1, vol/vol/vol) was used as developing solvent. Respective fractions were transmethylated in the presence of boron trifluoride/methanol complex.[21] Fatty acid methyl ester (FAME) of 2-monoacylglycerols and free fatty acids were subjected to gas chromatography.

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Supplementary data

Optimization of the acylation, kinetics studies of the **1**:**2** mixture acylation, detailed experimental procedures, characterization of products **4a**, **4c**–**d**, **5a**, **5c**–**d**, **6b**–**f**, **7b**–**f** and NMR spectra of all compounds are reported. Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.tet.2010.09.070. These data include MOL files and InChiKeys of the most important compounds described in this article.

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