Chemoenzymatic Synthesis of Enantiopure Structured Triacylglycerols

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Received 2 June 2008

Abstract: A highly efficient chemoenzymatic method for the synthesis of enantiopure ABC-type asymmetrically structured triacylglycerols has been developed starting from enantiopure (*S*)-solketal and involving two lipase steps.

Key words: asymmetrically structured triacylglycerols, regioselectivity, lipase, eicosapentaenoic acid, acyl migration

Structured triacylglycerols (TAG) constituting saturated, medium-chain fatty acids (MCFA) at the terminal *(sn*-1/*sn*-3) positions and long-chain, biologically active polyunsaturated fatty acids (PUFA) at the mid-position (*sn*-2) of the glycerol backbone have gained increased attention of scientists as dietary and health supplements.[1] Recently, a highly efficient synthesis of such structured MLM (medium-long-medium) type TAG possessing pure eicosapentenoic acid (EPA) or docosahexenoic acid (DHA) at the mid-position has been described by a two-step chemoenzymatic process.[2] Numerous beneficial effects on human health have been firmly established for the long-chain n-3 PUFA that are characteristic of marine fat.[3] These effects are almost exclusively attributed to EPA and DHA, the two most prevalent n-3 PUFA in marine fat.[4] Compound **1** (Figure 1) represents a structured TAG of the type described with capric acid at the end-positions and DHA at the mid-position.

Synthesis of such positionally labeled structured TAG by traditional synthetic organic chemistry methods requires a full regioselectivity control and can hardly be undertaken without multistep protection–deprotection processes. Owing to their regioselectivity, lipases are ideally suited as biocatalysts for preparing structured TAG.[5] By acting preferably or exclusively at the primary alcoholic positions of the glycerol backbone they may be employed to introduce fatty acids of certain type or composition at these positions by esterification or transesterification processes.[6] An immobilized *Candida antarctica* lipase (CAL)[7] was observed to display excellent regioselectivity toward the end-positions of glycerol using vinyl esters as acylating agents. This is based on a rapid, irreversible transesterification of glycerol using 1.25-fold stoichiometric amount of the vinyl esters of MCFA in dichloromethane at 0–4 °C. The lipase acted exclusively on the glycerol end-positions and excellent yields (≥ 90%) were obtained as based on chemically and regioisomerically pure material after recrystallization. It took the reaction only 3–5 hours to proceed to completion resulting in quantitative conversion into the desired 1,3-DAG with only traces of 1-monoacylglycerol (1-MAG) intermediate present throughout the reaction. The n-3 PUFA were subsequently introduced into the remaining mid-position of the symmetric 1,3-diacylglycerol key adducts, highly efficiently using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDAC) as a chemical coupling agent.[2]

The current work describes the design of synthesis of a novel type of structured TAG by a chemoenzymatic approach starting with optically pure solketal and involving two lipase steps. This is an enantiopure ABC-type asymmetrically structured TAG possessing two different types of pure saturated fatty acids at the *sn*-1 and *sn*-3 positions and a bioactive PUFA such as EPA or DHA at the *sn*-2 position of the glycerol backbone. In compound **2** (Figure 2) the structure of such asymmetrically structured TAG is revealed for EPA located at the *sn*-2 position, stearic acid at the *sn*-1 position, and capric acid at the *sn*-3 position of the glycerol moiety. This is the synthetic objective of the work described in the present report.

Such asymmetrically labeled ABC-type structured TAG may find value in various applications including screening for biological effects of individual fatty acids, possibly related to their location in stereospecific positions of the glycerol backbone. They may also become useful as chiral substrates to investigate lipase enantioselectivity

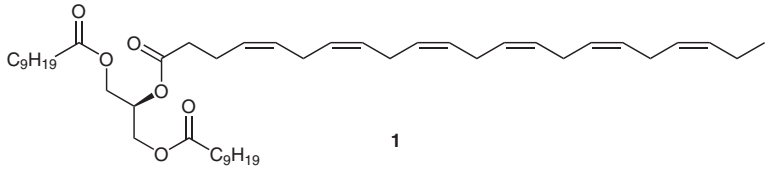

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Figure 1

SYNLETT 2008, No. 14, pp 2178–2182
Advanced online publication: 31.07.2008
DOI: 10.1055/s-2008-1077981; Art ID: D19508ST

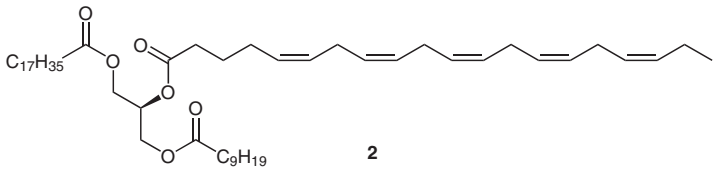
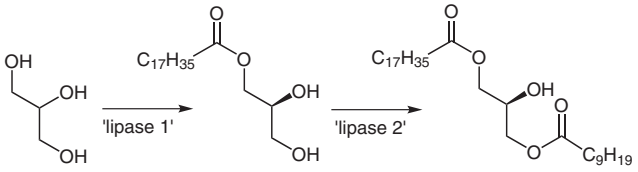


Figure 2

towards TAG, as standards, fine chemicals, and drug supplements. The methodology may also be utilized to introduce isotopically labeled fatty acids into predetermined positions of TAG and in design of prodrugs based on the glycerol framework.

It is pretty obvious that a synthetic route towards enantiopure ABC-type TAG would have to involve an enantiopure 1,3-DAG possessing all required regio- and stereochemistry as a key intermediate adduct. An ideal way to bring about synthesis of such enantiopure 1,3-DAG is by an all-enzymatic two-step process starting from nonsubstituted prochiral glycerol (Scheme 1).



Scheme 1 Hypothetical enzymatic two-step process to prepare asymmetric 1,3-DAG

The first step in such an approach requires a highly regio- and enantioselective lipase ('lipase 1' in Scheme 1) acting exclusively at the *sn*-1 position of glycerol to afford an enantiopure (*S*)-1-MAG adduct (or its *R*-enantiomer, depending on the enantiopreference of the lipase). A second lipase ('lipase 2' in Scheme 1) subsequently acts highly regioselectively or exclusively at the nonaccommodated *sn*-3 position to introduce a different fatty acyl moiety at that position to afford the asymmetric 1,3-DAG. For that lipase no enantioselectivity is needed.

Despite its simplicity this route turned out not to be very realistic after all. Lipases of the second type are certainly known, one of the most efficient being CAL as has been demonstrated in our previously described synthesis of structured MLM-type TAG.[2] But, there are apparently no reports on enantioselective acylations of nonsubstituted prochiral glycerol involving lipase and fatty acids or their esters to discriminate between the *sn*-1 and *sn*-3 positions, although there is a report of moderate enantioselectivity with vinyl benzoate.[8] There are, however, numerous reports on asymmetric biotransformations involving lipase and variously substituted glycerol derivatives by kinetic resolution.[9]

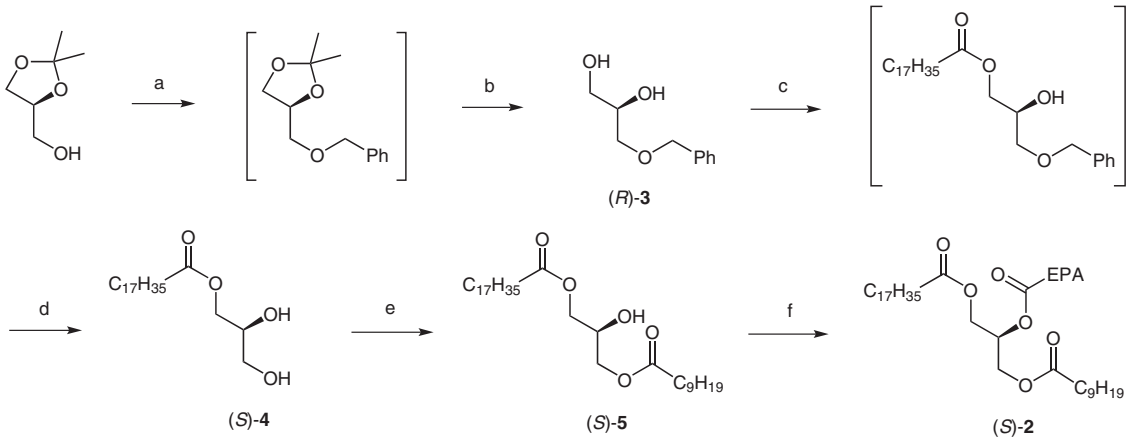
Lipases are known to act enantioselectively on TAG molecules, but usually their enantiopreference for the *sn*-1 or *sn*-3 positions is only moderate or relatively low.[10] Based on such lipase enantioselectivity there are reports on attempts to prepare asymmetric AAB- and ABC-type structured TAG.[11] Although moderate enantioselectivity was obtained in these attempts enantiopurities were nowhere close to what the current aims require. However, excellent enantioselectivity (>99% ee) was obtained in a recently reported enantioselective ethanolysis of homogeneous TAG using an immobilized *Rhizomucor miehei* lipase.[12] That lipase displayed strong enantiopreference for the *sn*-1 position of the TAG, but the resulting optically pure *sn*-2,3-DAG was obtained in only 60% chemical purity in a mixture with 2-MAG, unreacted TAG and glycerol.

An acyl group can be easily introduced to the *sn*-1 position of enantiopure (*R*)-solketal to afford the isopropylidene-protected MAG adduct using CAL and vinyl esters. However, the *sn*-1-MAG did not survive mild acidic conditions required for the deprotection of the isopropylidene moiety. This resulted in a mixture of approximately 90% 1-MAG and 10% 2-MAG, which is close to a reported equilibrium.[13]

A more extensive multistep protection–deprotection approach was clearly needed for the synthesis of (*S*)-**2**. The proposed synthetic route is based on a six-step chemoenzymatic process starting from optically pure (*S*)-solketal as is illustrated in Scheme 2.

The first four steps were needed for sorting out the stereochemistry and introducing stearic acid into the glycerol *sn*-1 position to afford a regioisomerically and optically pure 1-MAG (*S*)-**4**. This involves a benzyl ether protection of the free hydroxyl group, deprotection of the isopropylidene moiety, a highly regioselective introduction of pure stearic acid to the primary hydroxyl group of the resulting 1-*O*-benzyl-*sn*-glycerol (*R*)-**3** by CAL, and a catalytic hydrogenolysis of the benzyl protective group. The remaining part of the synthesis is rather straightforward by introduction of capric acid exclusively to the vacant primary *sn*-3 hydroxyl group by CAL providing the asymmetric regioisomerically pure 1,3-DAG key adduct (*S*)-**5**, and a subsequent introduction of EPA to its mid-position by chemical coupling.

Full enantiocontrol was secured by the use of commercially available enantiopure (*S*)-solketal as a chiral precursor.[14] Regioselective lipase was used to control the regiochemistry. The regiopurity, and therefore the enantiopurity, was maintained by mild conditions offered by the lipase acting at mild temperature. The good success is believed to predominantly relate to the enzyme displaying a superb regioselectivity and elimination of any acyl-migration side reaction.[2,5] The acyl migration is interrelated to various important factors including temperature, appar-



Scheme 2 *Reagents and conditions*: (a) NaH, THF, then BnBr; (b) 1 M HCl, H_2O–EtOH, reflux 30 min, 87% (2 steps); (c) vinyl stearate, CAL, CH_2Cl_2, r.t.; (d) H_2, 10% Pd/C, THF–hexane, 85% (2 steps); (e) vinyl capriate, CAL, THF, r.t., 85%; (f) EPA, EDAC, DMAP, CH_2Cl_2, r.t., 91%.

ently the most crucial single parameter, reaction rate, support material of enzyme, type of reaction, acyl donor, and reaction conditions.[2,13,15,16] The temperature was maintained sufficiently low to keep the acyl migration and lipase regioselectivity completely under control with the lipase still acting fast enough, since the acyl-migration process is clearly time-dependent. This was brought about by use of activated vinyl esters offering a fast and irreversible reaction.[2]

In the first step sodium hydride was used as a base in THF to introduce a benzyl protective group to the *sn*-3 position of the glycerol moiety by Williamson ether synthesis using benzyl bromide. The benzylated solketal adduct was not isolated but directly submitted to deprotection of the isopropylidene protective moiety using acidic aqueous ethanol. The resulting 3-*O*-benzyl-*sn*-glycerol adduct (*R*)-**3** was isolated by Kugelrohr distillation in vacuo in 87% overall yield.[17] Both (*R*)-**3** and its optical antipode (*S*)-**3** are commercially available.[18] The latter adduct has been used previously as a chiral precursor in synthesis of asymmetric enantiopure 2,3-DAG.[19]

The benzylated glycerol adduct (*R*)-**3** was subsequently acylated with vinyl stearate using the immobilized CAL at room temperature in dichloromethane. It took the reaction only 70 minutes to proceed to completion. The acylated benzyl ether adduct was not purified but directly introduced to Pd/C-promoted catalytic hydrogenolysis deprotection of the benzyl protective group in THF–hexane. As had been anticipated no acyl migration took place during this reaction as was firmly established by thorough investigations based on ^{1}H NMR spectroscopy at 400 MHz. The absence of acyl migration rules out possibilities that any losses in enantiopurity were taking place during these reactions. The overall two-step yield of the enantiopure 1-MAG (*S*)-**4** was 81% after crystallization from hexane.[20] This adduct along with its optical antipode has been previously prepared from D-mannitol by use of xanthen-9-ylidene protecting groups.[21]

The second enzymatic step involved the highly regioselective CAL a second time, this time to acylate exclusively at the *sn*-3 position of the 1-MAG (*S*)-**4** with vinyl capriate in dry THF at room temperature. Only 2–3 hours were needed to complete the reaction. This is in good agreement with our previous observation that only traces of 1-MAG were present during the two-step process when preparing the symmetric 1,3-DAG from glycerol suggesting that 1(3)-MAG, once formed, reacts significantly faster with the MCFA vinyl ester than glycerol under the reaction conditions used.[2] It should be kept in mind that the reaction conditions were different from the previous case, room temperature instead of 0–4 °C and, for solubility reasons, THF replacing dichloromethane. The enantiopure asymmetric 1,3-DAG adduct (*S*)-**5** was obtained in 85% yield after crystallization from methanol.[22] The specific optical-rotation value is extremely low for this type of compounds, $[\alpha]_D^{20}$ +0.02 (*c* 10, CH_2Cl_2), but further investigations by varying the solvent are under way.

The final step involved chemical coupling with EDAC in the presence of 4-dimethylaminopyridine (DMAP) to introduce pure EPA into the *sn*-2 position of adduct (*S*)-**5** to afford the asymmetrically structured ABC-type TAG final product (*S*)-**2** in 91% yield after purification by silica gel chromatography.[23] The reaction was conducted in dichloromethane at room temperature for twelve hours. Stoichiometric amount of EPA was used, 20% molar excess of EDAC and 0.4 equivalents of DMAP. As noticed before[2] no acyl migration was observed to take place during this reaction. This was further confirmed by detailed stability studies of 1,3-DAG using 400 MHz ^{1}H NMR spectroscopy under the reaction conditions of the coupling reaction in absence of EPA. The specific optical-rotation value of (*S*)-**2** was somewhat an order of magnitude higher than for its precursor, $[\alpha]_D^{20}$ +0.16 (*c* 10, CH_2Cl_2), but again, further investigations by varying the solvent are under way.

The enantiopurity of the compounds involved in this study has not been determined explicitly, but the fact that no acyl migration was detected at any stage of the reactions

implies that no depletions in enantiopurity were taking place during the reactions involved. Loss in enantiopurity would most certainly have to take place through acyl migration. The detection limits for such migration products by ^{1}H NMR spectroscopy (400 MHz) are estimated below 0.25% as based on careful intensive studies, whereas an equilibrium composition between 1(3)-MAG and 2-MAG is roughly 10% 2-MAG and 90% 1(3)-MAG.[13] The corresponding equilibrium composition for 1,3-DAG and 1(3),2-DAG is roughly 70% 1,3-DAG and 30% 1(3),2-DAG.[16] Therefore, losses in enantiopurity through lack of regiocontrol are rather unlikely, but this will be confirmed later by accurate measurements by chiral HPLC.

Acknowledgment

The University of Iceland Research Fund is acknowledged for financial support, Novozyme AS in Denmark for the lipase, and Olav Thorstad of Pronova Bicocare ASA in Norway for pure EPA. Dr. Sigridur Jonsdottir and Dr. Sigurdur V. Smarason at University of Iceland are acknowledged for high-resolution NMR and accurate MS measurements.

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(20) **Procedure for the Preparation of 1-*O*-Octadecanoyl-*sn*-glycerol [(*S*)-4]**

To a solution of 3-*O*-benzyl-*sn*-glycerol [(*R*)-**3**, 1.497 g, 6.73 mmol] and vinyl stearate (3.827 mg, 12.3 mmol) in CH_2Cl_2 (8.2 mL) was added immobilized CAL (99 mg). The resulting suspension was stirred at r.t. for approx. 70 min when TLC monitoring (EtOAc–PE, 1:1) indicated a complete reaction. The lipase preparation was separated by filtration and the solvent removed in vacuo on a rotary evaporator. The resulting residue was dissolved in THF (30 mL) without further purification followed by addition of *n*-hexane (70 mL) and Pd/C catalyst (370 mg). The reaction was performed in a PARR reactor under hydrogen pressure (5 bar) during which the product precipitated. When the reaction had proceeded to completion (about 2 h), THF was added until all the product had been dissolved. The catalyst was separated off by filtration by the aid of Celite and the solvent removed in vacuo on a rotary evaporator. The residue was redissolved in minimum amount of THF and a fourfold volume of *n*-hexane added. The resulting mixture was allowed to stand at r.t. overnight to afford the product as white crystals (2.057 g, 5.73 mmol) in 85% overall yield; mp 59.5–60.7 °C. $[\alpha]_D^{20}$ +2.43 (*c* 6, THF). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.21 (dd, *J* = 11.6, 4.8 Hz, 1 H, $OCOCH_2$), 4.15

(dd, J = 11.6, 6.0 Hz, 1 H, OCOCH_2), 3.96–3.90 (m, 1 H, CH_2CHCH$_2$), 3.72–3.67 (m, 1 H, CH_2OH), 3.63–3.57 (m, 1 H, CH_2OH), 2.52 (d, J = 5.2 Hz, 1 H, CHOH), 2.35 (t, J = 7.6 Hz, 2 H, CH_2COO), 2.08 (t, J = 6.0 Hz, 1 H, CH$_2$OH), 1.65–1.59 (m, 2 H, CH_2CH$_2$COO), 1.36–1.18 (m, 28 H, CH$_2$), 0.88 (t, J = 6.5 Hz, 3 H, CH$_2$CH_3). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 174.3, 70.3, 65.2, 63.3, 34.1, 31.9, 29.7 (5 C), 29.6 (2 C), 29.4 (2 C), 29.3, 29.2, 29.1, 24.9, 22.7, 14.1. FT-IR: 3150–3600 (br, OH), 2918 (vs, CH), 2849 (vs, CH), 1735 (vs, C=O) cm^{-1}. HRMS (APCI): *m/z* calcd for $C_{21}H_{42}O_4$ – OH: 341.3050; found: 341.3042 amu.

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(22) **Procedure for 3-*O*-Decanoyl-1-octadecanoyl-*sn*-glycerol [(*S*)-5]**

Immobilized *Candida antarctica* lipase (45 mg) was added to a 10 mL round-bottom flask containing a mixture of 1-octadecanoyl-*sn*-glycerol [(*S*)-**4**, 203 mg, 0.566 mmol] and vinyl decanoate (168 mg, 0.849 mmol) dissolved in THF (2 mL). The resulting suspension was stirred at r.t. for 2–3 h or until TLC monitoring (EtOAc–PE, 1:1) indicated a complete reaction. The lipase preparation was removed by filtration and the solvent removed in vacuo on a rotary evaporator to afford pure 3-*O*-decanoyl-1-octadecanoyl-*sn*-glycerol [(*S*)-**5**, 246 mg, 0.480 mmol] as a white crystalline material in 85% yield after recrystallization from MeOH; mp 53.4–54.0 °C; $[\alpha]_D^{20}$ +0.02 (*c* 10, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.20–4.06 (m, 5 H, CH$_2$CHCH$_2$), 2.47 (d, J = 3.6 Hz, 1 H, OH), 2.34 (t, J = 7.6 Hz, 4 H, CH$_2$COO), 1.68–1.59 (m, 4 H, CH_2CH$_2$COO), 1.36–1.18 (m, 40 H, CH$_2$), 0.87 (t, J = 6.8 Hz, 6 H, CH$_3$). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 173.9 (2 C), 68.4, 65.1 (2 C), 34.1 (2 C), 32.0, 31.9, 29.7 (5 C), 29.6 (2 C), 29.5 (2 C), 29.4 (3 C), 29.3 (2 C), 29.1 (2 C), 24.9 (2 C), 22.7 (2 C), 14.1 (2 C). FT-IR: 3300–3600 (br, OH), 2914 (vs, CH), 2849 (vs, CH), 1732 (vs, C=O), 1708 (vs, C=O) cm^{-1}. HRMS (APCI): *m/z* calcd for $C_{31}H_{60}O_5$ – OH 495.4408; found: 495.4412 amu.

(23) **Procedure for 3-*O*-Decanoyl-2-eicosapentaenoyl-1-octadecanoyl-*sn*-glycerol [(*S*)-2]**

To a solution of 3-*O*-decanoyl-1-octadecanoyl-*sn*-glycerol [(*S*)-**5**, 100 mg, 0.195 mmol] and EPA as free acid (66.0 mg, 0.218 mmol) in CH_2Cl_2 (2 mL) were added DMAP (20 mg, 0.16 mmol) and EDAC (50 mg, 0.26 mmol). The resulting solution was stirred on a magnetic stirrer hotplate at r.t. for 12 h. The reaction was disconnected by passing the reaction mixture through a short column packed with silica gel by use of Et_2O–CH_2Cl_2 (10:90). Solvent removal in vacuo afforded the pure product as a yellowish oil (141 mg, 0.177 mmol, 91% yield). $[\alpha]_D^{20}$ +0.16 (*c* 10, CH_2Cl_2). ^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.42–5.33 (m, 10 H, =CH), 5.32–5.23 (m, 1 H, CH$_2$CHCH$_2$), 4.29 (dd, J = 12.0, 4.4 Hz, 2 H, CH_2CHCH_2), 4.14 (dd, J = 12.0, 6.0 Hz, 2 H, CH_2CHCH_2), 2.86–2.78 (m, 8 H, =CCH$_2$C=), 2.34 (t, J = 7.6 Hz, 2 H, CH$_2$COO in EPA), 2.31 (t, J = 7.6 Hz, 4 H, CH$_2$COO), 2.14–2.04 (m, 4 H, =CCH_2CH$_2$ and =CCH_2CH$_3$), 1.70 (quint, J = 7.6 Hz, 2 H, CH_2CH$_2$COO in EPA), 1.64–1.55 (m, 4 H, CH_2CH$_2$COO), 1.34–1.20 (m, 40 H, CH$_2$), 0.97 (t, J = 7.6 Hz, 3 H, CH$_3$ in EPA), 0.88 (t, J = 6.8 Hz, 6 H, CH$_3$ in C_{18}). ^{13}C NMR (100 MHz, $CDCl_3$): δ = 173.3 (2 C), 172.6, 132.0, 128.9, 128.8, 128.6, 128.3, 128.2, 128.1, 128.0, 127.8, 127.0, 69.0, 62.1 (2 C), 34.0 (2 C), 33.6, 31.9, 31.8, 29.7 (5 C), 29.6 (2 C), 29.5, 29.4 (2 C), 29.3 (2 C), 29.2 (3 C), 29.1, 26.5, 25.6 (3 C), 25.5, 24.8 (2 C), 24.7, 22.7, 22.6, 20.5, 14.3, 14.1 (2 C). FT-IR: 3013 (s, CH), 2925 (vs, CH), 2854 (vs, CH), 1745 (vs, C=O) cm^{-1}. HRMS (APCI): *m/z* calcd for $C_{51}H_{88}O_6$ + NH_4: 814.6919; found: 814.6913 amu.