Measuring Stereoselectivity in Lipase-Catalyzed Acidolysis Reactions by Ultra-High Resolution ^{13}C Nuclear Magnetic Resonance

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ABSTRACT: Elucidating the stereoselectivity of lipases in synthetic reactions of triacylglycerols has hitherto been carried out using traditional analytical techniques to determine the composition of the reaction products. These methods are laborious and are not always appropriate for analysis of certain triacylglycerol types. A direct method, utilizing a stereospecific deuterium-labeled triacylglycerol substrate, has been developed where the stereoisomeric composition of the reaction product is determined by ultra-high resolution ^{13}C nuclear magnetic resonance (NMR) spectroscopy. Through lipase-catalyzed transesterification of deuterium-labeled trilauroylglycerol with oleic acid, chemical shifts were induced in the ^{13}C NMR spectrum by the deuterium atom and olefinic double bonds, enabling unambiguous stereospecific assignment of triacylglycerol species. By this method of analysis, we found an effect of the degree of reaction conversion on the extent of stereoisomerism in the triacylglycerol product. Stereoselectivity was greatest (for *sn*-1) with lipase from *Rhizomucor miehei*. Lipases from *Rhizopus niveus, Candida rugosa, Carica papaya,* and the cutinase from *Fusarium* sp. were also found to exhibit stereoselectivity, with preference for either *sn*-1 or *sn*-3 acyl exchange.

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KEY WORDS: Acidolysis, chemical shifts, chiral, cutinase, deuterium, lipase, NMR, stereoselectivity, triacylglycerol, triglyceride.

Asymmetric acylglycerol molecules (containing different moieties in positions *sn*-1 and *sn*-3) exist as *R* and *S* stereoisomers. In lipase-catalyzed hydrolysis of di- and triacylglycerols, it has been observed that certain lipases exhibit a preference for one stereoisomer over the other (1–4). Stereoselectivity has also been reported in lipase-catalyzed acidolysis reactions under certain conditions of low water activity, particularly with the lipase from *Rhizomucor miehei*, making possible the synthesis of chiral triacylglycerols (5). A number of methods for the stereochemical analysis of triacylglycerols have been described (6–10). Lipase stereoselectivity in microaqueous reactions is currently determined by indirect chemical analysis. For example, the triacylglycerol products of a reaction can be quantified after partial "hydrolysis" by Grignard reagents and derivatization with chiral reagents to form diastereomers, which are then separated chromatographically. These analytical methods cannot be applied in all situations or only with difficulty, particularly when short-chain acyl groups are present. Here we describe a new method for analyzing stereochemistry by ultra-high resolution ^{13}C nuclear magnetic resonance (NMR) spectroscopy, using a remote carbon–carbon double bond as a reporter group modifying the chemical shift of a carbonyl group. The principle is illustrated by application to the stereochemical analysis of lipase-catalyzed triacylglycerol interesterifications.

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MATERIALS AND METHODS

Materials. All chemicals, substrates, and solvents were purchased in the highest available purity from the Sigma-Aldrich group (Poole, United Kingdom). Lipase (EC 3.1.1.3) from *Rhizopus niveus* [Lipase N (4,500 Lipase Units/g)] was purchased from Amano Pharmaceutical Co. (Nagoya, Japan). Lipase from *R. miehei*, supported on Duolite resin [Lipozyme® IM (100,000 LU/g)] was a gift from Novo Nordisk A/S (Bagsværd, Denmark). Lipase from *Candida rugosa* [Lipase OF360 (360,000 LU/g)] was purchased from Meito Sangyo (Tokyo, Japan). Lipase from *Carica papaya* was extracted from papain obtained from Sigma-Aldrich Ltd. Cutinase from *Fusarium solani pisi* was donated by the Biotechnology unit of Unilever Research (Vlaardingen, The Netherlands). Macroporous polypropylene enzyme support (Accurel EP100, particle size distribution 200–400 μm) was purchased from Akzo Nobel GmbH (Obernburg, Germany). All other solvents and chemicals were of analytical reagent (AR) quality and purchased from Fisher Scientific Ltd. (Loughborough, United Kingdom) or Sigma-Aldrich Ltd.

Immobilization of lipases onto Accurel EP100. All lipases were used in immobilized form. Unless supplied already immobilized, the lipases were adsorbed onto Accurel EP100, following the method described by Bosley and Peilow (11).

Preparation of C. papaya *latex.* The method described by Giordani *et al.* (12) was followed. The latex was used directly as an acidolysis catalyst and had a hydrolysis activity of 5500 LU/g (on tributyrin).

Synthesis of stereospecifically labeled triacylglycerol substrates. A known method for the production of enantiomerically pure triacylglycerols was adapted so that an atom of

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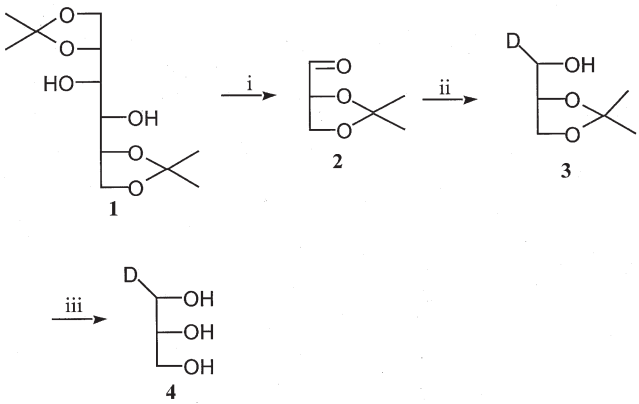


Reagents: i, $Pb(OAc)_4$; ii, $Na[^2H_4]BH_4$; iii, H^+/H_2O

SCHEME 1

deuterium ($^2H_1$) was incorporated nonstereospecifically onto the *sn*-3 glyceryl carbon (13) (Scheme 1). 1,2:5,6-Di-*O*-isopropylidene-D-mannitol [**1**, 5 g] was dissolved in cold ethyl acetate (100 mL) and oxidized by stepwise addition of lead (iv) tetra-acetate (8.7 g) over 20 min. The product, 1,2-*O*-isopropylidene-D-glyceraldehyde [**2**, 3.6 g] was recovered by distillation, dissolved in ethanol (50 mL), and reduced with sodium [$^2H_4$] borohydride (0.35 g, 98% atom ^2H) to 1,2-*O*-isopropylidene-D-glycerol (**3**, 1.1 g). The protecting group was removed by acid hydrolysis to yield *sn*-[3-$^2H_1$]-glycerol (**4**, 0.6 g). The deuterium atom was assigned to the *sn*-3 carbon by application of IUPAC rules on the stereospecific numbering of triacylglycerols (14). Trilauroyl-*sn*-[3-$^2H_1$]-glycerol was produced by complete acylation of **4** with lauroyl chloride in the presence of pyridine. Mass spectrometry data: *m/z* 658 (molecular ion + NH_4^+); compares with *m/z* 657 (molecular ion + NH_4^+) for unlabeled trilaurin, indicating monodeuteration.

Enzyme-catalyzed reactions of triacylglycerol and free fatty acid. All reactants and solvents were equilibrated to a water activity of 0.11, at ambient temperature (20°C), over saturated lithium chloride in a sealed vessel for at least 7 d, as described by Valivety *et al.* (15). It is assumed that, provided sufficient time is allowed for equilibration, the water activity of the sample equals that of the salt solution. The acidolysis reactions of trilauroyl-*sn*-[3-$^2H_1$]-glycerol (73 mg; 114 µmol) and oleic acid (97 mg; 343 µmol) were performed and analyzed according to the method described in Chandler *et al.* (5). From the analysis of data, a degree of reaction conversion (dc) was calculated, where:

$$dc = 100 \times \% \text{ oleic acid in total triacylglycerol} / \% \text{ oleic acid in total triacylglycerol at equilibrium} \quad [1]$$

^{13}C NMR. ^{13}C NMR spectra at high resolution were obtained using a Bruker ACP400 spectrometer (Karlsruhe, Germany). Spectra were obtained at the highest available resolution (0.015 Hz/data point), aided by a sample longer than usual (4 cm), temperature control (298 K), minimal decoupling power, and Lorentzian to Gaussian resolution enhancement.

Stereoselectivity factor (SF). Using Microsoft (Redmond, WA) Excel Solver, integration data from ^{13}C NMR spectra were quantitatively correlated with triacylglycerol composition data from gas chromatography analyses. A measure of the extent of stereoselectivity and the configuration of the products was obtained from the equation:

$$SF = OLaLa^*/(OLaLa^* + LaLaO^*) \quad [2]$$

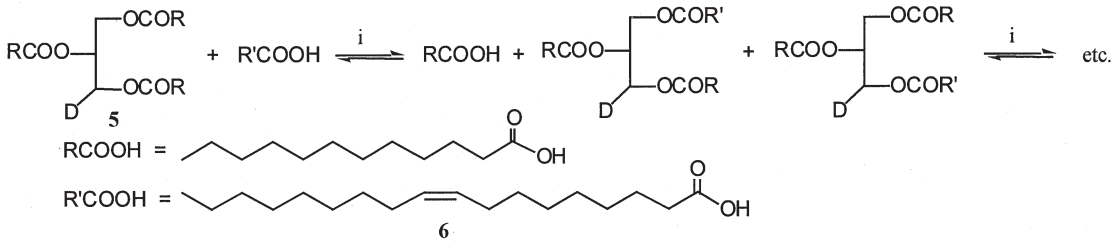
where O is oleate, La is laurate, and * represents the deuterated glyceryl position (*sn*-3). An SF value of 1.00 represents total selectivity for *sn*-1 (*S*), 0.00 represents total selectivity *sn*-3 (*R*), and 0.50 represents a racemic triacylglycerol composition.

RESULTS AND DISCUSSION

Method development. In seeking a simpler and more direct method for determining the stereochemistry of lipase-catalyzed interesterification in triacylglycerols, the use of stereospecifically labeled glycerol was envisaged. A triacylglycerol such as trilaurin **5** (Scheme 2), prepared from such a labeled glycerol, might undergo lipase-catalyzed fatty acid exchange against a different acid, oleic acid **6** for example, to give enantiomeric products arising from exchange of the *sn*-1 and *sn*-3 fatty acyl groups, respectively. Elucidation of the stereochemistry of this process could then be based on determining the ratio of fatty acid exchange at the deuterated and nondeuterated positions (Scheme 2).

One option considered for determining this ratio was by the use of MS, relying on C–C bond fragmentation between the 1,2- and 2,3-positions in the glycerol component. This fragmentation would give ions that would consist of nondeuterated and monodeuterated species in a ratio directly related to the stereospecificity of fatty acid exchange. However, the required fragmentation was difficult to achieve and produced the required ions only in very low abundance and with low reproducibility.

As an alternative, the use of NMR was explored in the anticipation that a combination of deuteration shifts and



Reagents: i, lipase

SCHEME 2

substituent shifts might reveal the positional specificity of fatty acid exchange. This hope was realized when the carbonyl region of the ^{13}C NMR spectra of products derived from deuterated trilaurin by interesterification against oleic acid was analyzed at ultra-high resolution. Stereochemical analysis was made possible by the operation of two effects: chemical shift changes arising from long-range effects of the olefinic double bond in oleyl components, and deuteration shifts affecting acyl substituents at the labeled *sn*-3 position. The laurate-oleate shift differences arise from the effects of the dipoles at the ends of the oleyl double bond, and it should be noted that the influence of the reporter olefinic bond is transmitted over eight carbon–carbon single bonds. However, this method of stereochemical characterization relies on the presence of detectable shift differences between the reaction substrates and products, and therefore would not be suitable for the study of reactions involving the exchange of long-chain saturated acyl groups.

^{13}C NMR spectra at high resolution were obtained. The chemical shift of a given carbonyl resonance was determined by the environment (acyl group attached to a primary or secondary center) and by the two structural factors noted above, namely, the nature of the side chain and the position of the carbonyl group relative to the deuterated position.

In Figure 1 is shown the carbonyl region of the ^{13}C NMR spectrum of the product of interesterification of trilaurin with oleic acid catalyzed by the lipase from *R. miehei*. In the region δ 172.7–173.18 ppm, the carbonyl carbon atom resonances fall into five groups. At the lowest field, the signal **a** is attributable to the lauryl groups attached to the deuterated *sn*-3 position. The deuteration shift is +7 ppb and is unexpectedly positive. This can be attributed to a small reduction in γ-*gauche* shielding at the carbonyl carbon atom, which arises from the shorter C–D bond distance compared with the C–H bond distance (16). Confirmation that this assignment is correct was obtained by determining the spectrum of a mixture of deuterated and nondeuterated trilaurin in unequal proportions. From the relative intensities of the signals attributable to deuterated and nondeuterated material, it was possible to assign them unambiguously. The resonances in region **b** arise from lauryl carbonyl groups attached to the *sn*-1 position, and magnetically equivalent lauryl groups attached at the *sn*-3 position in undeuterated glycerol remaining in the deuterated sample used for preparation of the trilaurin. In region **c** are seen resonances attributable to oleyl groups attached to the *sn*-3 position. The chemical shift is the net result of a positive deuteration shift superimposed on a negative shift arising from the long-range effect of the unsaturation in the oleyl side chain (17–19). Region **d** encompasses the resonances attributable to oleyl groups attached at the *sn*-1 position and oleyl groups attached to the *sn*-3 position in material derived from residual unlabeled glycerol. Resonances attributable to acylation at the 2-position are seen in region **e** near δ 172.74. Only a single resonance is seen as the lipase from *R. miehei* does not catalyze exchange at the *sn*-2 position. It should be noted that the range of chemical shifts of signals attributable to acyl groups attached at the *sn*-1 and *sn*-3 positions is only 50 ppb.

The percent deuteration, the percent conversion, and the stereoselectivity of the interesterification are readily derived from the integrals of the signals in the regions **a** to **e** (Fig. 1). Thus, the stereoselectivity is given by the ratio OLaLa*/(OLaLa* + LaLaO*) and the asterisk indicates that the corresponding residue is attached to the deuterated position. The other species potentially present are LaLaLa*, OLaO*, LaLaLa, OLaLa, and OLaO. The proportions of these were adjusted, using Microsoft Excel Solver, to give the best fit to the five resolved signals. For the example shown in Figure 1, the calculated stereoselectivity is 0.38, the extent of deuteration is 81%, and there have been 56% single replacements of lauryl groups

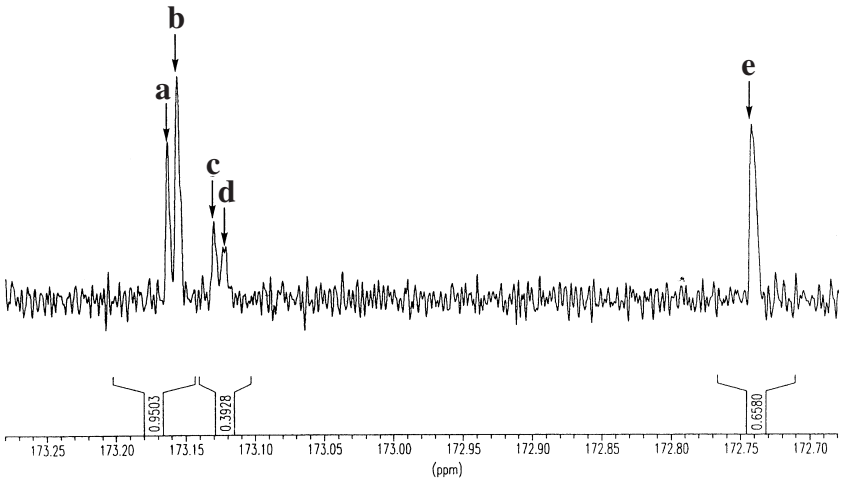


FIG. 1. ^{13}C Nuclear magnetic resonance (NMR) spectrum, in the range δ 172.7–173.25 ppm, of the product of interesterification of trilauroyl-*sn*-[3-$^{2}H_1$]-glycerol **5** against oleic acid **6** catalyzed by the lipase from *Rhizomucor miehei*. Signals **a**–**e** have been attributed to chemical shifts according to the vicinity of the carbonyl group to unsaturated bonds or deuterium atoms. These are discussed fully within the text.

TABLE 1
Stereoselectivity of Reactions of Trilauroylglycerol and Oleic Acid Catalyzed by *Rhizomucor miehei*[a] Lipase at Varying Degrees of Conversion (dc)

	Triacylglycerol composition (normalized to 100%)					
dc[b] (%)	LaLaLa	LaLaO/ OLaLa	OLaO	OOO	SF	% ee[d] (configuration)[e]
14	80	18	2	0	1.00	100 (*S*)
25	64	31	4	1	1.00	100 (*S*)
34	53	40	7	1	0.86	72 (*S*)
44	42	48	10	1	0.73	46 (*S*)
69	21	52	22	4	0.67	34 (*S*)

[a]This lipase was prepared using recombinant DNA technology: The genetic coding for the lipase was transferred from the source organism to a host, *Aspergillus niger.*
[b]Percent conversion defined as 100 × [oleate in triacylglycerol/oleate in the triacylglycerol at equilibrium].
[c]Stereoselectivity factor = OLaLa*/(OLaLa* + LaLaO*).
[d]% ee of OLaLa [note: % ee = 100 × (2SF − 1) where SF = stereoselectivity factor and ee = enantiomeric excess].
[e](*S*) stereoisomer arises from *sn*-1 exchange.

by oleyl groups. An indication of the consistency of the method is that the deuterium content as deduced from the ^{13}C NMR data agrees well with the ratio determined by integration of the peaks attributable to the glycerol protons in the ^{1}H NMR spectrum of the same sample (data not shown).

In Tables 1 and 2 are shown the results of interesterifications between trilaurin and oleic acid catalyzed by the lipases from *R. miehei, R. niveus*, *C. rugosa,* and *C. papaya*, and by the cutinase from *F. solani pisi*.

Lipase from R. miehei. Under the conditions applied, the lipase from *R. miehei* exhibited high stereoselectivity (for position *sn*-1). This agreed with previous findings (5). However, in using the earlier analytical methods it was not possible to determine the effect of the degree of reaction conversion. At low conversions, stereoselectivity was essentially complete. However, as the interesterification reaction proceeded toward equilibrium, the stereochemical integrity of the interesterification product was lowered. Because at equilibrium the concentrations of enantiomers must be equal (e.g., OLaLa = LaLaO), the stereoselectivity is time dependent, as observed.

Lipase from R. niveus. At very low conversion, a high degree of selectivity for *sn*-1 was observed for lipase from *R. niveus*. At a degree of conversion of >60% the product stereoisomerism is lost; this also agrees with previous experiments (5). In comparing stereoselectivities between different enzymes, the extent of conversion must be taken into account. However, when stereoselectivities are compared at the same percent conversions (interpolating where necessary), it can be seen that the lipase from *R. miehei* does indeed exhibit a higher stereoselectivity than the lipase from *R. niveus*. It should be noted, however, that the reaction conditions had been optimized for catalysis by *R. miehei* lipase only.

Lipases from C. rugosa *and* C. papaya*, and* Fusarium *cutinase.* These enzymes all have similar, but low, stereoselectivi-

TABLE 2
Stereoselectivity of Reactions of Trilauroylglycerol and Oleic Acid Catalyzed by Lipases (or cutinase) from Various Sources

		Triacylglycerol composition (normalized to 100%)					
Source organism[a]	dc[b] (%)	LaLaLa	LaLaO/ OLaLa	OLaO	OOO	SF	% ee[d] (configuration)[e]
R. niveus	7	88	10	2	0	0.97	94 (*S*)
R. niveus	63	27	47	27	0	0.53	6 (*S*)
C. rugosa	55	37	42	18	3	0.44	12 (*R*)
C. papaya	49	45	34	18	3	0.47	6 (*R*)
F. solani pisi	23	68	29	5	1	0.36	28 (*R*)
F. solani pisi	52	36	47	15	2	0.42	16 (*R*)

[a]Lipases were expressed by the native organism. Organisms include *Rhizopus niveus, Candida rugosa, Carica papaya,* and *Fusarium solani pisi.*
[b]Percent conversion defined as 100 × [oleate in triacylglycerol/oleate in the triacylglycerol at equilibrium].
[c]Stereoselectivity factor = OLaLa*/(OLaLa* + LaLaO*).
[d]% ee of OLaLa [note: % ee = 100 × (2SF − 1)].
[e](*S*), (*R*) stereoisomers arise from *sn*-1 and *sn*-3 exchange, respectively. See Table 1 for other abbreviations.

TABLE 3
A Comparison of Data from Different Stereoselectivity Assays for Selected Lipases

	Hydrolysis of trioleoylglycerol (monolayer technique)[b]			Acidolysis of trilauroyl-*sn*-[3-$^2H_1$]-glycerol		
Lipase source[a]	dc	% ee	*sn*-1/3	dc	% ee	*sn*-1/3
R. miehei	6	82	1	14	100	1
R. arrhizus[c]	6	55	1	—	—	—
R. niveus[c]	—	—	—	7	94	1
C. rugosa	6	14	1	55	12	3
F. solani pisi	6	72	3	28	16	3

[a]Organisms include *Rhizomucor miehei, Rhizopus arrhizus, Rhizopus niveus, Candida rugosa,* and *Fusarium solani pisi.*
[b]Rogalska, Cudrey, Ferrato, and Verger method (1).
[c]No direct comparison is available. The results from tests with lipases from related *Rhizopus* species are given.

ties at the degrees of conversion measured. With these catalysts the stereopreference was found to be toward the *sn*-3 position, and strong *sn*-3 selectivity has been reported for both the *C. papaya* latex lipase and the *Fusarium* cutinase (6,20). However, there is a notable discrepancy in the stereopreference assignment for *C. rugosa* lipase, which has been shown to exhibit *sn*-1 selectivity in the hydrolysis of triacylglycerols (1,3). An insufficient number of experiments have been conducted with *C. rugosa* lipase to be confident of our determination of *sn*-3 selectivity in the acidolysis reaction.

In Table 3 a comparison has been made (where data are available) between the hydrolytic method of stereoselectivity determination used by Rogalska *et al*. (1) and our acidolysis method. As already discussed, the degree of conversion can affect the enantiomeric excess obtained, which limits the correlation of data, and the acidolysis exchange conditions were only optimized for *R. miehei* lipase. Nevertheless, *C. rugosa* data withstanding, there appears to be general agreement between methods for the limited data available. Further validation of the acidolysis technique with a wider range of lipases and reaction conditions is necessary.

Further splittings of the NMR signals. In some experiments, the NMR signals observed (as in Fig. 1) showed further fine splittings of the order of 3 ppb (Fig. 2), particularly in cases, as here, corresponding to high conversions. These arise through the same through-bond dipolar processes that bring about separation of the lauryl and oleyl carbonyl signals at a given glycerol site, except that in this case the perturbation is caused by chain variations at the other sites transmitted through the glycerol backbone. Although the exact signal assignment has not been confirmed, the observed shifts are approximately consistent with the attenuation factors deduced previously; therefore, all have the same sign (16,18). These extra shifts have not been reported previously and offer a potential method for assessing the chain distribution in an unknown di- or triacylglycerol mixture.

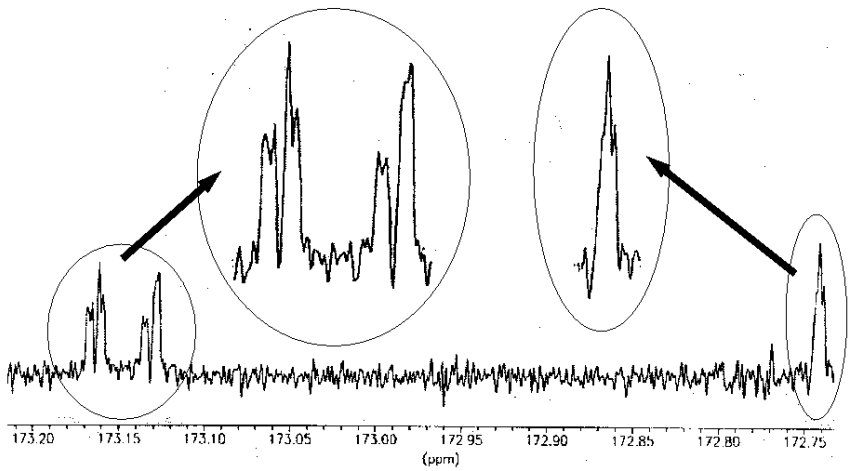


FIG. 2. ^{13}C NMR spectrum, in the range δ 172.7–173.25 ppm, of the product of interesterification of trilauroyl-*sn*-[3-$^2H_1$]-glycerol **5** against oleic acid **6** catalyzed by the lipase from *Rhizomucor miehei.* The spectrum shows fine structure attributable to long-range effects on individual carbonyl resonances of acyl groups attached at other positions, and these have been enlarged to show the detail. The signals have the same chemical shifts as in Figure 1. See Figure 1 for abbreviation.

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