New fluorogenic triacylglycerol analogs as substrates for the determination and chiral discrimination of lipase activities

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Abstract   A new type of fluorogenic and isomerically pure 1(3)-O-alkyl-2,3 (3,2)-diacyl glycerols was synthesized that can be used as substrate for the determination of lipase activities. These compounds contain a fluorescent pyrene acyl chain and, as a potent quencher of pyrene fluorescence, a trinitrophenylamino acyl residue. In their intact form, the fluorogens show only low fluorescence intensity. Upon lipase-induced or chemical hydrolysis of the substrates, however, the fluorophore and quencher separate from each other. This leads to a gradual increase in pyrene fluorescence, reflecting the time-dependent progress of lipolysis and, under substrate saturation conditions, lipase activity. This lipase assay is continuous and does not require separation of substrate and reaction products. Short- and long-chain homologues as well as optical isomers of the fluorogenic alkyldiacyl glycerols were hydrolyzed by pancreatic lipase, hepatic lipase, and lipoprotein lipase at highly different rates depending on the substrate or enzyme preparation and source (e.g., postheparin plasma or cultured cells). It is proposed that a useful set of enantiomeric and/or homologous substrates in combination with appropriate reaction media might be applied to the selective determination of a lipase in a mixture of lipases, e.g., hepatic and lipoprotein lipase in PHP, for medical diagnostics.—Duque, M., M. Graupner, H. Stütz, I. Wicher, R. Zechner, F. Paltauf, and A. Hermetter. New fluorogenic triacylglycerol analogs as substrates for the determination and chiral discrimination of lipase activities. *J. Lipid Res.* 1996. **37:** 868–876.

Supplementary key words   hepatic lipase • pancreatic lipase • lipoprotein lipase • fluorescent lipids • albumin-lipid complex • lipase assay • alkyldiacyl glycerols • pyrene-glycerolipids • post-heparin plasma • glycerolipid synthesis • stereoselectivity

Lipases are found in biological material originating from animals, plants, and microorganisms (1, 2). Determination of enzyme activities is routinely necessary in the screening of natural lipase sources for the characterization of recombinant proteins, and also for monitoring enzyme activities during isolation and purification of lipases. Additionally, lipases play an important role in human (patho-) physiology. Determination of their activities is important for the diagnosis of hyperlipidemia (3), acute pancreatitis (4), and other conditions. Finally, lipases are chiral catalysts for the preparation of enantiomerically pure chemicals required for the preparation of biologically active compounds, such as pharmaceuticals, odorants, pesticides, etc. (5).

A great number of methods are available for assaying lipase activities. The most commonly used techniques include titrimetric (1), radioactive (2), or photometric and fluorescence methods (6–11). Most of these techniques suffer from several shortcomings, such as poor reproducibility, lack of sensitivity, or the production of radioactive waste. Many involve time-consuming and cost-intensive procedures. The chemical structures of some of the fluorogenic substrates that are commercially available are quite unlike those of the natural substrates and may not be useful for the assay of lipases of various origins.

Herein we describe the synthesis and application of a new generation of fluorogenic alkyldiacyl glycerols that are useful as substrates for the determination of lipase activities in aqueous media and organic solvents. In particular, they can be used for routine lipase determination in body fluids, as even in complex biological samples such as blood plasma, lipase activities can be

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Abbreviations: TNP, trinitrophenylamino;   MPLC, medium-pressure liquid chromatography; RET, resonance energy transfer; PHP, post-heparin plasma; VLDL, very low density lipoprotein; HDL, high density lipoprotein; BSA, bovine serum albumin; HPLC, high pressure liquid chromatography; TLC, thin-layer chromatography.

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analyzed with high accuracy. Finally, it is demonstrated that the enantiomers and homologues of the new compounds are hydrolyzed to different degrees by different lipases. In combination with specific reaction conditions this might eventually allow the discrimination of several lipases in a given sample.

MATERIALS AND METHODS

Standard chemicals were from Merck, Darmstadt, Germany. Trinitrophenylamino (TNP)-sulfonic acid and TNP-aminododecanoic acid were purchased from Sigma, Deisenhofen, Germany. Bovine serum albumin was from Sigma. Fatty alcohols were from Nu-Chek-Prep, Elysian, MN. Optically pure *sn*-1,2- and *sn*-2,3-isopropylideneglycerols were obtained from Bachem, Buchs, Switzerland. Pyrene fatty acids were from Molecular Probes, Eugene, OR. The fluorogenic lipase substrates and a kit for the determination of lipase activities are available from PROGEN, Heidelberg, Germany.

Lipases

Lipoprotein lipase was isolated from bovine milk as described by Zechner (12). In addition, a commercial lipase from Sigma was tested. Rat liver perfusate was
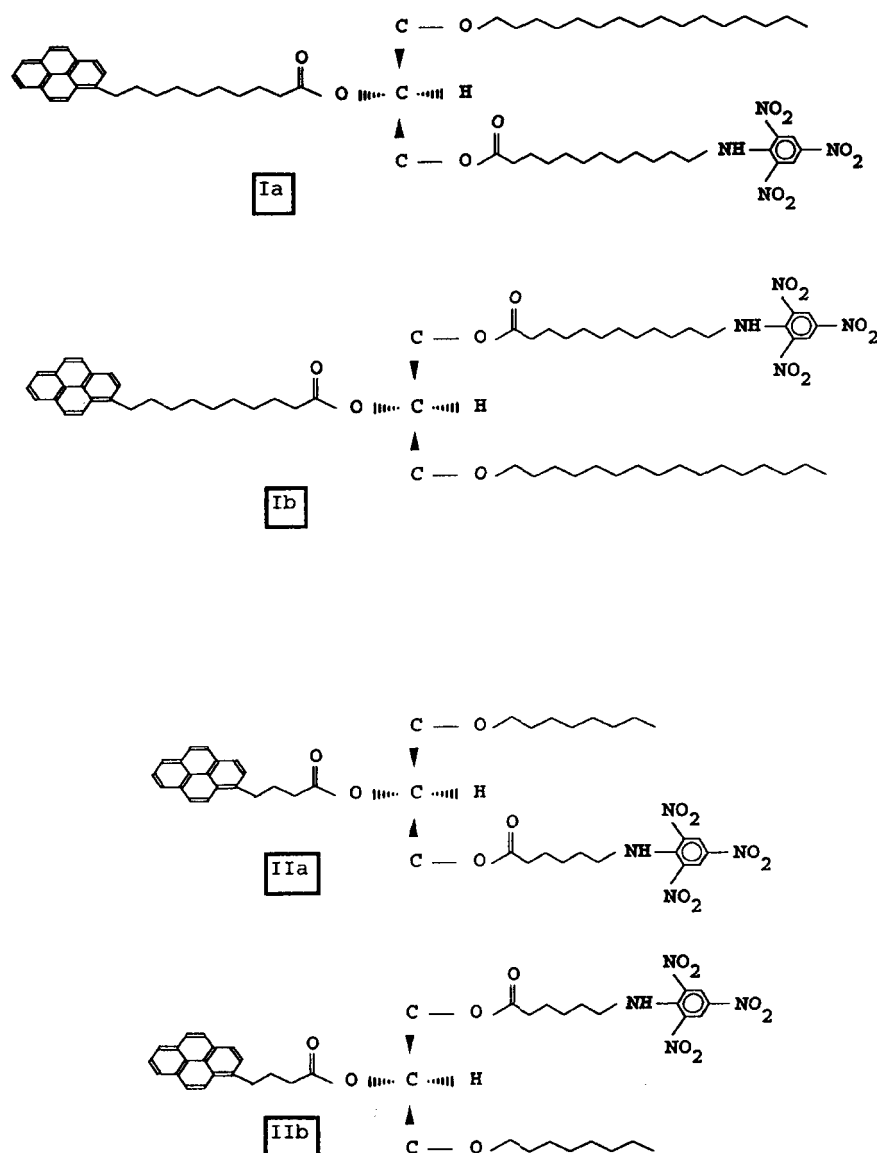
Fig. 1. Chemical structures of long (Ia, Ib)- and short (IIa, IIb)-chain enantiomers of fluorogenic alkyldiacyl glycerol. Ia = 1-O-hexadecyl-2-pyrenedecanoyl-3-trinitrophenylaminododecanoyl-*sn*-glycerol. Ib = 1-Trinitrophenylaminododecanoyl-2-pyrenedecanoyl-3-O-hexadecyl-*sn*-glycerol. IIa = 1-O-octyl-2-pyrenebutanoyl-3-trinitrophenylaminohexanoyl-*sn*-glycerol. IIb = 1-Trinitrophenylaminohexanoyl-2-pyrenebutanoyl-3-O-octyl-*sn*-glycerol.

used as a source of hepatic lipase. Porcine pancreatic lipase (50,000 units/mg protein) and colipase were from Sigma. PHP was taken 10 min after heparinization of the donor (60 U/kg).

Thin-layer chromatography (TLC)

TLC analysis was carried out using alumina plates coated with Kieselgel 60 from Merck. Fluorescent spots were detected under a UV-lamp. Organic material was detected by charring after spraying with 50% H_2SO_4.

Optical spectroscopy

Absorption measurements were carried out on a spectrophotometer U-3210 from Hitachi. Fluorescence

R_1 = ALKYL

R_2—C(=O) = PYRENE - ACYL (FLUOROPHORE)

R_3—C(=O) = TRINITROPHENYLAMINO - ACYL (QUENCHER)

Fig. 2. Synthesis of fluorogenic alkyldiacyl glycerols (1(3)-O-alkyl-2-pyreneacyl-3(1)-trinitrophenylaminoacyl-sn-glycerols).

measurements were performed using a fluorometer RF 540 from Shimadzu. Pyrene fluorescence emission spectra were recorded using an excitation wavelength of 342 nm (emission and excitation slits were set 10 and 5 nm, respectively).

Fluorescence lipase assay

Bovine serum albumin (BSA) (0.4 mg, fraction V, fatty acid-free) was dissolved in 3 ml PBS-buffer. A solution of the substrate in tetrahydrofuran (6 nmol in 25 μl) was added to the BSA solution under stirring at 37°C. The final substrate concentration was 2 μM. The solubilized substrates were stored overnight at 4°C for equilibration before use. Enzyme samples were added to 3 ml of substrate solution and rates of lipolysis were determined from the continuous increase in fluorescence intensity at 378 nm (excitation 342 nm, slit width 10 nm each) on a Shimadzu spectro-fluorometer RF-540 for several minutes. All measurements were performed at 37°C.

pH-stat method

Lipolysis was monitored using a continuous pH-stat method (pH-stat from Metrohm, Herisau, Switzerland) (13). Tributyroyl glycerol (300 μl) was emulsified in 10 ml buffer (2 mM Tris-HCl, pH 7.4, 2 mM sodium deoxycholate, 0.9% NaCl). The emulsion was magnetically stirred and temperature-controlled (37°C) in a pH-stat vessel. Lipolysis was started by injection of varying amounts of pancreatic lipase in the presence of colipase (0.21 μg/μg pancreatic lipase). The pH was kept constant at 7.4 by the addition of 0.05 M NaOH.

NMR spectra

^{1}H-NMR spectra were recorded at 300 MHz on a Bruker MSL 300 NMR-spectrometer (Bruker, Karlsruhe, Germany), using tetramethylsilane as an internal reference. Spectra were run in $CDCl_3$. Multiplicities are reported as singlet (s), doublet (d), triplet (t), or multiplet (m).

Synthesis of 1-O-hexadecyl-2-pyrenedecanoyl -3-trinitrophenylaminododecanoyl-sn-glycerol (Ia)

1-O-Hexadecyl-3-O-triphenylmethyl-sn-glycerol was synthesized starting from 2,3-isopropylidene-sn-glycerol, as described by Hermetter and Paltauf (14).

1-O-Hexadecyl-2-pyrenedecanoyl-3-O-triphenylmethyl-sn-glycerol was synthesized from 1-O-hexadecyl-3-O-triphenylmethyl-sn-glycerol (137 mg, 245 μmol) and pyrenedecanoic acid (100 mg, 268.8 μmol), dissolved in 5 ml of water-free methylene chloride. After subsequent addition of dimethylamino pyridine (100 mg, 818.5 μmol) and dicyclohexylcarbodiimide (85 mg, 412 μmol), the reaction mixture was stirred at 45°C overnight. The precipitate was removed by filtration over a glass sinter
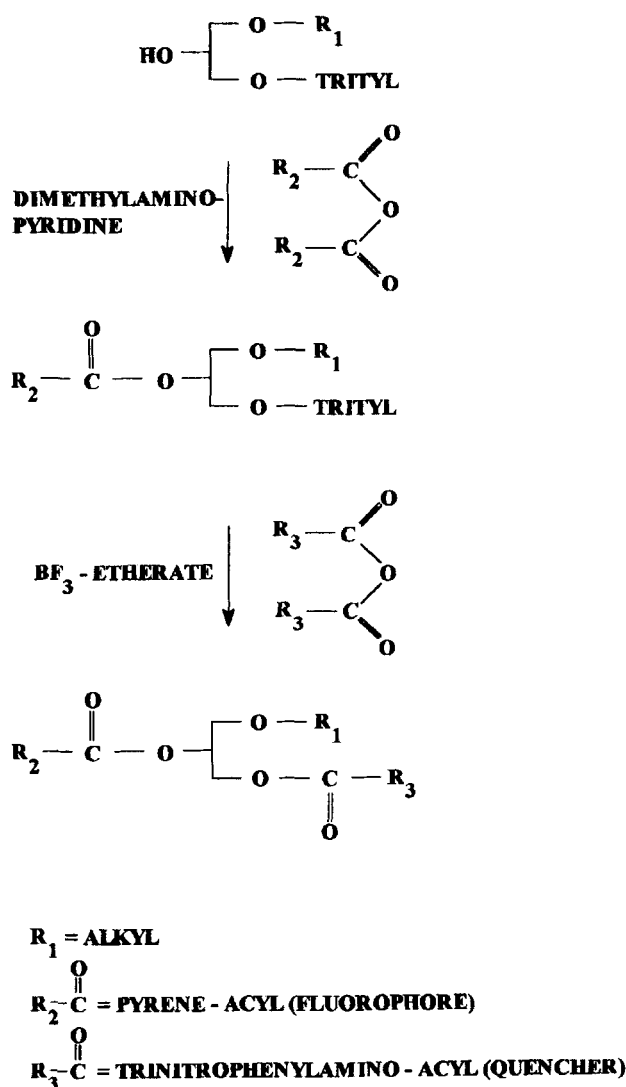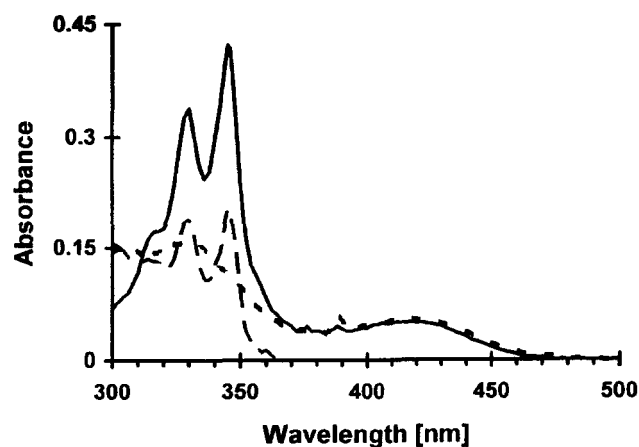
Fig. 3. Absorption spectra of the labeled triacylglycerol analogues (Ia–IIb, see Fig. 1) (—), containing pyrene alkanoic acid (— —), and trinitrophenylaminoalkanoic acid (- - -).

plate. The filtrate was washed twice with 1 ml water and dried over sodium sulfate. The solvent was removed under a nitrogen stream and the crude product was dissolved in petroleum ether and purified by medium-pressure liquid chromatography (MPLC) on silica gel (1.5 × 30 cm column). The product was eluted, using a petroleum ether–diethyl ether gradient. Fractions of 5 ml were collected. The fractions containing the pure compound were combined and the solvent was removed under vacuum. The pure alkylacyltrityl glycerol (43 mg, 47.1 µmol) was obtained in 19.2% yield. It showed a single spot, R_f 0.9 on TLC (solvent: petroleum ether–diethyl ether 8:2, v/v).

Trinitrophenylaminododecanoic acid anhydride was prepared from the free acid (300 mg, 703.6 µmol) and dicyclohexylcarbodiimide (73 mg, 353.8 µmol) in 2 ml water-free methylene chloride as described by Selinger and Lapidot (15).

The carboxylic acid anhydride (280 mg, 335 µmol) and hexadecyl-pyrenedecanoyl-trityl-glycerol (150 mg, 164 µmol) were dissolved in 2 ml water-free methylene chloride. After addition of 500 µl BF₃–etherate complex (325 mg BF₃), the reaction mixture was stirred at 0°C for 3 h. The dark yellow solution was poured onto 10 ml chloroform–methanol–25% NH₃ 65:35:5 (per vol), followed by washing with two 2-ml portions of methanol–water 1:1 (v/v). The solvent was removed under vacuum and the crude product was dissolved in petroleum ether for MPLC purification on a silica gel column (1.5 × 30 cm). The pure compound was eluted, using a petroleum ether–diethyl ether gradient. Fractions (5 ml each) containing the pure compound were combined and the solvent was removed under vacuum. The pure product (90 mg, 82.8 µmol, 50.5% yield) showed a single

spot, R_f 0.5, on TLC (solvent: petroleum ether–diethylether 8:2, v/v).

According to absorption measurements, the molar ratio of pyrene to the trinitrophenylamino residue was close to 1.0. Extinction coefficients of pyrene decanoic acid and trinitrophenylamino decanoic acid chromophores in ethanol were 42000 at 342 nm and 7000 at 414 nm, respectively.

¹H-NMR (CDCl₃): ppm 9.03 (3 H, s, aromat); 8.31–7.86 (10 H, m, aromat); 5.12 (1 H, m, sn-2-CH); 4.29, 4.19 (4 H, m, sn-3-CH₂); 3.73 (2 H, m, H₂C–CH₂–O); 3.34 (2 H, t, H₂C–CO); 2.98 (2 H, t, H₂C–CO); 2.35 (4 H, t, CH₂–CH₂–CO); 1.86 (2 H, m, CH₂–aromat); 1.62–1.01 (48 H, m, CH₂–CH₂–O, CH₂–CH₂–aromat, (CH₂)₂₂); 0.90 (3 H, s, –CH₃).

Synthesis of 1-trinitrophenylaminododecanoyl-2-pyrenedecanoyl-3-O-hexadecyl-sn-glycerol (Ib)

1-O-Triphenylmethyl-3-O-hexadecyl-sn-glycerol was synthesized starting from 1,2-isopropylidene-sn-glycerol as described by Hermetter and Paltauf (14).

1-O-Triphenylmethyl-2-pyrenedecanoyl-3-O-hexadecyl-sn-glycerol was synthesized from the corresponding triphenylmethyl-hexadecyl-sn-glycerol by acylation with pyrenedecanoic acid as described above for the enantiomeric compound.

Triphenylmethyl-pyrenedecanoyl-hexadecyl-glycerol (204 mg, 223 µmol), trinitrophenylaminododecanoic acid anhydride (380 mg, 455 µmol) and BF₃–etherate (675 µl, 438 mg BF₃) were reacted in 4 ml water-free methylene chloride as described for compound Ia. After preparation and MPLC purification, 59 mg (54 µmol, 24.3% yield) of the pure compound was obtained. Its absorption and ¹H-NMR spectra were identical to those of its enantiomer (see Ia, above).
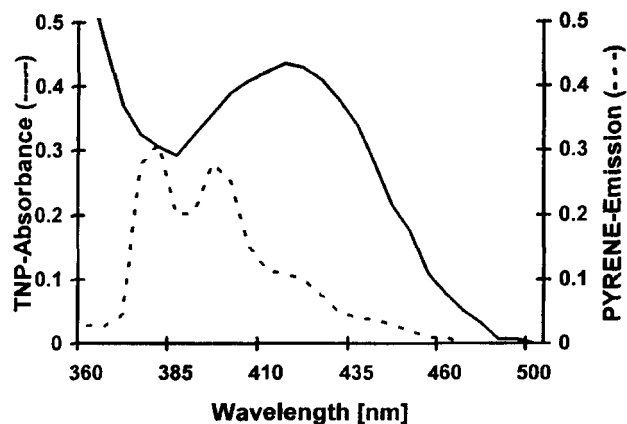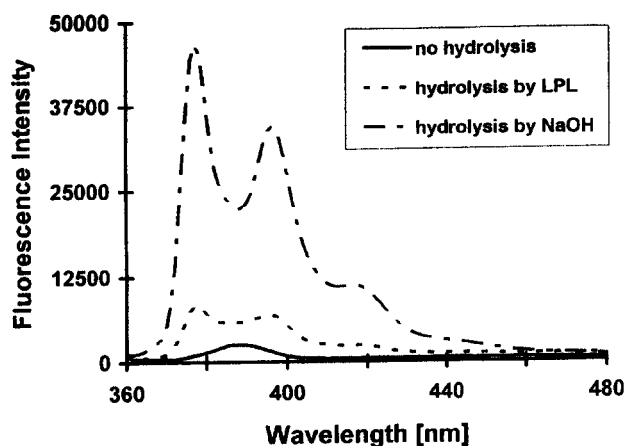

Fig. 4. Spectral overlap of quencher (TNP) absorption and fluorophore (PYRENE) emission in fluorogenic alkyldiacyl glycerols; TNP, trinitrophenylamino. The substrate (2 nmol/ml) was solubilized in ethanol.

Fig. 5. Effect of hydrolysis on the fluorescence of fluorogenic alkyldiacyl glycerol. Samples contained 2 nmol lipid/ml in phosphate-buffered saline. Fluorescence spectra were measured 20 min after addition of 10 N NaOH (100 µl/ml; (− · −) or lipoprotein lipase (6.9 µg partially purified enzyme, see reference 12, (- - -) at 37°C.

Synthesis of 1-O-octyl-2-pyrenebutanoyl -3-trinitrophenylaminohexanoyl-*sn*-glycerol (IIa)

1-O-Octyl-3-O-triphenylmethyl-*sn*-glycerol was synthesized, starting from 2,3-isopropylidene-*sn*-glycerol, as described by Hermetter and Paltauf (14).

Compound IIa was prepared by the same procedure as that for Ia. Starting from 50 mg (112 µmol) 1-O-octyl-3-O-trityl-*sn*-glycerol, 3.5 mg (25.7% yield in the last reaction step) pure IIa was obtained after MPLC. It showed a single spot, R_f 0.5, on TLC (solvent: petroleum ether–ether 8:2, v/v).

The absorption spectrum of IIa revealed a pyrene/trinitrophenylamino chromophore ratio of 1.0.

Synthesis of 1-trinitrophenylaminohexanoyl -2-pyrenebutanoyl-3-O-octyl-*sn*-glycerol (IIb)

1-O-Triphenylmethyl-3-O-octyl-*sn*-glycerol was synthesized, starting from 1,2-isopropylidene-*sn*-glycerol, as described by Hermetter and Paltauf (14).

Compound IIb was prepared by the same procedure as that for IIa. Starting from 40 mg (56 µmol) 1-O-octyl-2-pyrenebutanoyl-3-O-trityl-*sn*-glycerol, 10.5 mg (11.4 µmol, 20% yield in the last reaction step) pure IIb was obtained after MPLC. The final product showed the same behavior on TLC and the same spectral properties as those of compound IIa.

RESULTS

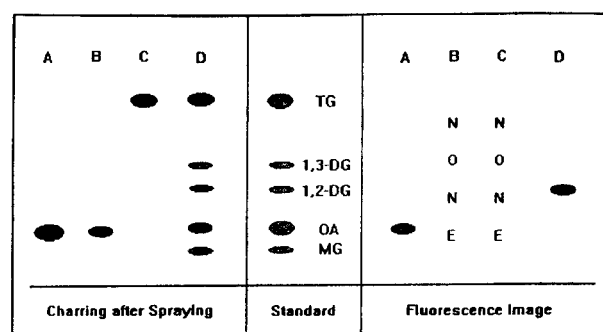### Chemical synthesis of fluorogenic alkyldiacyl glycerols

The newly synthesized fluorogenic alkyldiacyl glycerols (**Fig. 1**) are characterized by a number of structural features. They are composed of a fluorescent acyl chain

on the glycerol's secondary hydroxyl group and a quencher fatty acid esterified with one of the primary hydroxyl groups. The other primary oxygen of glycerol forms an ether bond with an alkyl group that cannot be released by hydrolytic cleavage. A similar spectroscopic principle has been used for the design of a phospholipase substrate (16). The respective compound is a phosphatidylethanolamine analog containing the fluorophore and the quencher covalently bound to an acyl chain and the amino residue of the polar head group, respectively.

The fluorogenic alkyldiacyl glycerols were synthesized starting from the corresponding, optically pure 1-O-alkyl and 3-O-alkyl-*sn*-glycerols (14) (**Fig. 2**). Tritylation of the primary hydroxyl function leading to alkyltritylglycerol was followed by acylation with pyrenealkanoic acid anhydride leading to alkylacyltrityl-*sn*-glycerol. Replacement of the trityl group by a trinitrophenylaminoalkanoic acid residue in the presence of boron trifluoride gave the final alkyldiacyl glycerol. The final reaction step is based on a procedure described for the synthesis of mixed-acid phosphatidylcholines from 1-O-trityl-2-acyl-*sn*-glycerophosphocholine (17). This is a very efficient method for the synthesis of isomer-free vicinal diacyl glycerolipids, as exchange of a trityl for an acyl group in a one-pot reaction avoids acyl migration and thus formation of positional isomers. After this procedure, the obtained optical isomers of short- and long-chain alkyldiacyl glycerols were stereochemically pure.

Characterization

Stereochemical purity of the fluorogenic triacyl glycerol analogues could be assessed by HPLC analysis (18) of diastereomers obtained after derivatization of the corresponding diradyl glycerols with (R+)

Fig. 6. TLC analysis of fluorogenic alkyldiacyl glycerol (Ib, see Fig. 1) and its lipolytic degradation products after hydrolysis by lipoprotein lipase. A: pyrenedecanoic acid; B: trinitrophenylaminododecanoic acid; C: fluorogenic alkyldiacyl glycerol; D: products of lipolysis of fluorogenic alkyldiacyl glycerol after preincubation (20 min) with lipoprotein lipase (11.5 µg partially purified enzyme, see reference 12) (for sample preparation see Materials and Methods). Standards: triolein (triacylglycerol, TG), 1,3-diacylglycerol (1, 3-DG), 1,2-diacylglycerol (1,2-DG), oleic acid (OA), monoacylglycerol (MG).
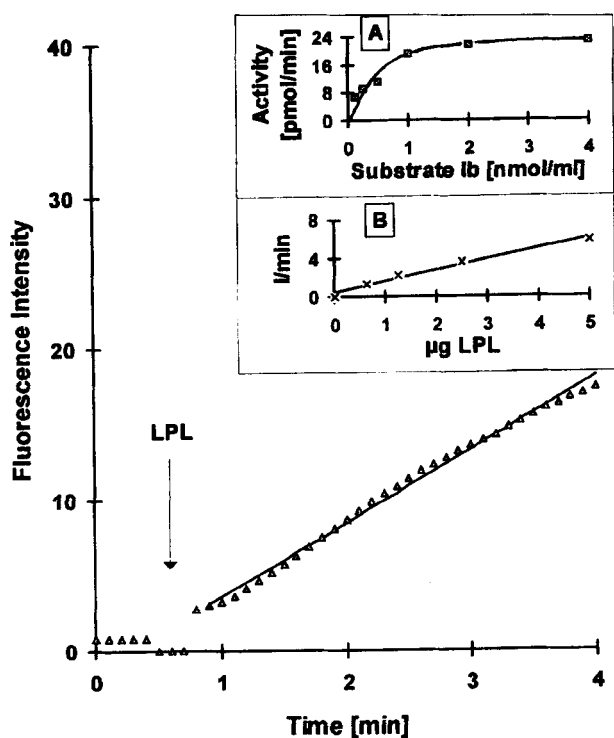
Fig. 7. Fluorescence assay for the determination of lipoprotein lipase activity. Enzyme activity was determined from the time-dependent increase in fluorescence intensity after addition of partially purified lipase (3 μg) to an aqueous dispersion of fluorogenic alkyldiacyl glycerol (Ib, see Fig. 1) in the presence of albumin (see Materials and Methods).

phenylethylisocyanate, and by the strict stereospecificity of the lipases from *Rhizopus arrhizus* and *Candida rugosa* towards one enantiomer of the long-chain substrates (G. Zandonella, F. Paltauf, and A. Hermetter, unpublished results).

Figure 3 shows the absorption spectrum of the labeled glycerolipid analogue Ia (see Fig. 1). It is precisely equal to the sum of the spectra of its components (pyrenealkanoic acid, trinitrophenylaminoalkanoic acid) at equimolar concentrations.

Pyrene fluorescence of the intact lipid molecules is very low, as the pyrene emission spectrum overlaps very efficiently with the absorption spectrum of the TNP residue (**Fig. 4**). Thus, pyrene fluorescence is quenched to a large extent by intramolecular resonance energy transfer (RET) (19) to TNP. From studies on the excimer fluorescence of 1,2- and 1,3-dipyreneacyalkyl glycerols it is known that these compounds in organic solvent and detergent micelles show an extended conformation such that the hydrophobic chains are not in close vicinity to each other (20). Even if we assume such a conformation for the new lipids I and II, TNP and pyrene must be located within the critical distance required for RET between this donor–acceptor pair, as effective pyrene quenching is observed in the same media.

Enzymatic and chemical hydrolysis

Hydrolysis of the fluorogenic lipids by NaOH or lipoprotein lipase releases the labeled acyl chains from the glycerol backbone in position 1 (3) and 2 (NaOH) and 1 (3) (lipase). If the hydrolysis products are effectively diluted in the reaction medium, pyrene fluorescence is dequenched and its emission intensity increases (**Fig. 5**). TLC analysis of the reaction mixture originating from the substrate in the presence of lipoprotein lipase or NaOH shows that the observed fluorescence increase is, in fact, a consequence of hydrolysis and is not due to any physical effect of the additives. **Figure 6** shows that the starting compound C can be well separated from its potential degradation products (see standard). The starting compound does not show any fluorescence, but only the yellow color originating from the TNP-aminoalkanoyl residue.

For lipase assays, the fluorogenic substrates were solubilized in aqueous buffer in the presence of equimolar amounts of fatty acid-free bovine serum albumin. Under these conditions the lipase activities could be determined with high sensitivity in a very reproducible manner. Hydrolysis by lipoprotein lipase leads to the formation of alkylacyl and alkyl glycerol, the latter being the final hydrolysis product, as the O-alkylether bond is not hydrolyzable. Within short reaction times (5 min) the primary ester bonds linking the TNP-amino fatty acid to glycerol are cleaved preferentially. Thus, the increase of fluorescence intensity during short reaction times after addition of lipase predominantly reflects the release of the quencher acyl chain from the glycerol backbone. Fluorescence detection of the TLC pattern after 20 min reveals the formation of fluorescent alkylpyreneacyl glycerol (due to release of the TNP acyl residue)
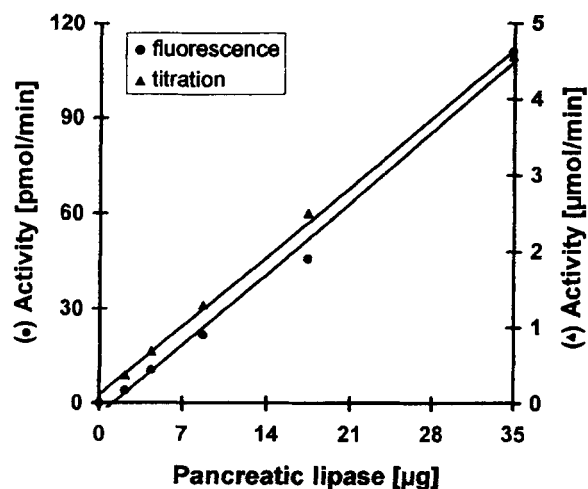

Fig. 8. Comparison of the fluorescence assay and the pH-stat method. Lipase activities in the presence of colipase (0.21 μg/μg pancreatic lipase) were determined, using tributyrin (pH-stat method, see Materials and Methods) or alkyldiacylglycerol Ib in BSA-containing PBS buffer (fluorescence assay).

TABLE 1. Activities of isolated animal lipases and lipases in human PHP toward fluorogenic alkyldiacyl glycerols

Lipases or Lipase Source	Lipase Activity			
	Short-Chain Substrate		Long-Chain Substrate	
	sn-1 Acyl	sn-3 Acyl	sn-1 Acyl	sn-3 Acyl
	pmol/ml/min			
Lipoprotein lipase;	117.34 ± 6.69	5.71 ± 1.76	129.50 ± 3.04	7.54 ± 0.25
Hepatic lipase	4.37 ± 0.24	1.35 ± 0.35	15.38 ± 2.04	1.10 ± 0.08
Pancreatic lipase	5.76 ± 0.22	9.18 ± 0.36	3.42 ± 0.05	0.60 ± 0.10
Human plasma	3.15 ± 0.35	1.55 ± 0.32	0.12 ± 0.02	0.02 ± 0.02
Human post-heparin plasma	17.4 ± 0.45	3.56 ± 0.09	7.36 ± 0.10	0.70 ± 0.04

Fluorogenic alkyldiacylglycerol (2 nmol/ml) was solubilized in the presence of fatty acid-free bovine serum albumin (2 nmol/ml) in PBS buffer, pH 7.4. Lipase was added to the substrate (assay volume: 2 ml) and the process of lipolysis was followed by the time-dependent increase of fluorescence intensity for 5 min. Enzyme activities (pmol/ml/min) were determined using a calibration curve obtained from an unquenched pyrene lipid standard (hexadecyl-pyrenedecanoyl-trityl-glycerol). Lipases: lipoprotein lipase (3 µg, from Sigma), hepatic lipase (40 µl, rat liver perfusate), pancreatic lipase (40 µg, from Sigma, plus 9 µg colipase from porcine pancreas, from Sigma), human (post-heparin) plasma (30 µl). Substrates: 1(3)-O-alkyl-2-pyreneacyl-3(1)-trinitrophenylaminoacyl-sn-glycerols (see Fig. 1).

and a lower amount of pyrenealkanoic acid released from position 2 of glycerol.

Hydrolysis of the alkyldiacyl glycerols by lipase leads to a time-dependent increase in fluorescence intensity, from which enzyme activity can be determined continuously within a few minutes (**Fig. 7**) under conditions of non-limiting substrate concentrations (insert A of Fig. 7 shows a plot of reaction rate versus substrate concentration). From a calibration curve obtained with an unquenched pyrene lipid (alkylacyltrityl glycerol), the reaction rates in pmol/min can be calculated. The continuous increase in fluorescence intensity due to substrate hydrolysis reflects the amount of fluorescent reaction product (hexadecyl-pyrenedecanoyl-glycerol) that can be isolated after extraction of the incubation mixture with chloroform–methanol 2:1 (v/v). Thus, the rate of lipolysis as determined from the continuous increase in fluorescence intensity is equal to the rate of separation of the quencher fatty acid from the substrate. Enzyme activities show a linear relationship when plotted against enzyme concentration (Fig. 7, insert B). The same linear dependence of enzyme activity on enzyme mass was obtained by the established pH-stat method and the fluorescence technique, although the absolute reaction rates measured are different (**Fig. 8**). An example is shown for porcine pancreatic lipase. The pH-stat technique uses an aliphatic triglyceride, namely tributyroyl glycerol, as a substrate that is susceptible to hydrolysis by lipases and esterases. The fluorescence substrate Ib is a long-chain triglyceride analog containing aromatic substituents at the ω-ends of two hydrophobic chains. As a consequence, it is hydrolyzed more slowly by lipases. On the other hand, it is absolutely stable in the presence of esterases. The lower reaction rates

observed with the fluorogenic substrates are overcompensated by the high sensitivity and reproducibility of the fluorescence assay.

Table 1 summarizes the analysis of various lipases and of lipase activity in human plasma. All lipases show considerable stereoselectivities both for the long- and short-chain substrate enantiomers, although the extent of stereoselectivity depends on the chain length of the substrate. In general, the sn-1 acyl isomers of the fluorescent alkyldiacyl glycerols are the preferred enantiomeric substrates for the enzymes under investigation, except for porcine pancreatic lipase. The latter lipase preferably hydrolyzes the sn-3 acyl enantiomer of the short-chain, but not of the long-chain lipids. Activities of porcine pancreatic lipase were determined in the presence of colipase. The presence of this cofactor stimulates its activity severalfold (data not shown). Lipoprotein lipase is more active and shows similar stereopreference in the presence of the long-chain and with the short-chain substrate. Activity and stereo-preference of hepatic lipase towards the long-chain alkyldiacyl glycerols is higher than that towards the short-chain analogues.

Lipolytic activity in human plasma from healthy donors without heparinization can be determined reliably with the short-chain substrate, but is very low when the long-chain lipid is used as a substrate. Plasma lipase activity after heparinization of the donor is much higher, reflecting the presence of lipoprotein lipase and hepatic lipase released from the arterial walls or their resident organs. Differences in lipase activities between different postheparin plasma samples were similar using the fluorogenic long-chain substrate or ³H-labeled triolein. The latter (aliphatic) substrate showed much higher

rates. However, the fluorescence test was at least as sensitive and much more reproducible (data not shown) (M. Duque, I. Wicher, R. Zechner, and A. Hermetter, unpublished results).

DISCUSSION

In this paper the synthesis and application of new fluorogenic triacylglycerol analogs is described for the analysis of lipases of animal and human origin. Activities were determined of isolated lipoprotein lipase from bovine milk, hepatic lipase from rat liver perfusate, porcine pancreatic lipase, and of lipase activities in PHP, the latter being composed of at least two components: lipoprotein lipase and hepatic lipase. Both enzymes are responsible for extracellular lipolysis of different lipoprotein classes (lipoprotein lipase for chylomicrons and very low density lipoprotein (VLDL), hepatic lipase for high density lipoprotein (HDL) and others) (3, 21). In living systems, impairment of extracellular lipid catabolism and uptake by cells may occur (3) if these enzymes are either present in too low amounts or impaired in function due to structural abnormalities or inhibitory effects.

The contribution of each of the two lipases to the observed total lipase activity of PHP as determined using the long-chain substrates Ia–b is yet unknown. Possible effects due to interferences between both enzymes in our assay system are currently being subjected to further investigation.

The hydrolytic activity of plasma toward the short-chain triglyceride analogues IIa–b might be due, in part, to the presence of low amounts of serum esterases (22). Esterase from porcine liver has already been shown to be active on this substrate. Nevertheless, the combined use of short- and long-chain substrates for the analysis of serum lipase activity could be advantageous in discriminating the different enzymes (lipoprotein lipase and hepatic lipase) under specific reaction conditions, as all these enzymes behave differently toward homologous and enantiomeric derivatives of the new substrates.

The new assay system should also be applicable to the analysis of different lipases that can be found in serum only under pathological conditions, e.g., in the course of destructive and inflammatory processes (e.g., pancreatitis, tumors). In the case of acute pancreatitis it might provide an effective method for the specific (owing to the stereo-preference of the lipase) and reliable analysis of lipase originating from the diseased organ.

Several lipases act on substrates other than triacylglycerols and analogues. In particular, hepatic lipase, and to a lesser extent lipoprotein lipase, show phospholipase A_1 activity (23). Many lipases, including hormone-sensitive lipase, also hydrolyze diacylglycerols (2). Fluorogenic phospholipids and diacylglycerols carrying the same labels as the alkyldiacyl glycerols described here are currently being tested as substrates for the respective enzymes.

The majority of the known techniques for the determination of lipase activities have in common the use of detergents as solubilizers of the natural, radioactive, or chromogenic substrates that are mostly hydrophobic. Thus, these assays give information about lipase activities on detergent–substrate mixed micelles, that do not necessarily mimic physiological conditions. It was our main concern to maintain "native conditions" when analyzing lipases using the new substrates. This may become especially important if lipases are to be determined in the presence of lipoproteins (of blood plasma) or in living cells.

We found that the fluorogenic alkyldiacyl glycerols were readily dispersed in aqueous buffer, when fatty acid-free albumin was present. Under such conditions, activities of isolated lipoprotein lipase, hepatic lipase, porcine pancreatic lipase, and PHP could be determined within minutes in a highly reproducible manner. The physico-chemical characterization of the putative albumin–lipid complexes that constitute this unique form of the substrate is currently under way. Preliminary results showed that the size of the respective particles is very similar to that of the lipid-free protein (8 nm diameter; M. Duque, N. Maurer, O. Glatter, and A. Hermetter, unpublished results).

The method described in this paper can easily be performed using an inexpensive fluorometer composed of a light source (xenon lamp), equipped with two filters (instead of monochromators) for wavelength selection and a detection system (photomultiplier). For the quantitative determination of very large numbers of samples (several hundreds) in a short time, a fluorescence plate reader can also be used.

The new method for the assay of lipase activities has the advantage of being continuous, efficient, and reproducible. It is essentially based on the following features: triacylglycerol-analogue substrates, potential for enzyme discrimination by substrate homologues or enantiomers, appropriate solubilization of lipophilic substrates in water for testing biological samples under nondisturbing conditions, and applicability to instrumentation that can be used in the biochemical as well as in the routine clinical laboratory. The fluorogenic substrates I–II show much lower lipase activities as compared to aliphatic triglycerides used for the pH-stat (tributyrin) or radioactive method ([^{3}H]triolein). Despite this difference in reaction rates, the fluorescence method has the advantage of being at least as sensitive, but much more reproducible and much faster. When

the long-chain compounds (I) are used as substrates the assay is lipase-specific (in contrast to the tributyrin-based pH-stat method). Radioactivity is avoided. The new substrates allow for a very simple lipase assay that can be applied to a large variety of reaction media such as aqueous buffers with or without detergent, organic solvent, monolayers at the air–water interface, or biological systems (e.g., lipoproteins or cells). Thus, we expect that they will find wide application in scientific as well as in routine laboratory practice.▣▣

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