Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Molecular Catalysis B: Enzymatic 22 (2003) 255–269
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Inhibition of digestive lipases by 2-oxo amide triacylglycerol analogues

George Kokotos*

Laboratory of Organic Chemistry, Department of Chemistry, University of Athens, Panepistimiopolis, Athens 15771, Greece

Accepted 5 December 2002

Abstract

Potent and specific inhibitors of digestive lipases are of interest because they contribute to the understanding of the molecular mechanism of lipase action, and they may also find medical applications. In this review, results obtained by our group concerning the design and synthesis of 2-oxo amide triacylglycerol analogues will be discussed, and a study of their inhibition effect on pancreatic and gastric lipases will be presented.

Keywords: Gastric lipase; Inhibition; 2-Oxo amides; Pancreatic lipase; Synthetic inhibitors

1. Introduction

Lipases are ubiquitous enzymes [1] which are found in animals, plants [2], fungi [3] and bacteria [4]. In 1958, lipases were defined in kinetic terms, based on the "interfacial activation" phenomenon, i.e. on the increase in the activity, which occurs when a partially water-soluble substrate becomes water-insoluble [5]. The recently determined 3D structures of lipases show an α/β hydrolase fold as well as a nucleophilic elbow where the catalytic serine is located [6]. Some, but not all, lipases contain a "lid" controlling access to their active site. However, both the "interfacial activation" phenomenon as well as the structural features described above are not suitable criteria for classifying specific esterases as lipases. Thus, lipases may be redefined as carboxylesterases acting on long-chain acylglycerols [7].

Lipases are enzymes that have attracted much attention for the following reasons:

(a) They are flexible biocatalysts for the acylation or deacylation of a wide range of unnatural substrates [8]. They have been widely employed by organic chemists to catalyze hydrolysis, alcoholysis, esterification and transesterification of triacylglycerols (TAGs) and analogues, thereby taking advantage of their fatty acid specificity, regio- and stereoselectivity [9].
(b) In humans and higher animals, pancreatic and gastric lipases are essential enzymes for efficient fat digestion [10]. The hydrolysis of dietary TAGs by these enzymes to monoacylglycerols and free fatty acids is a necessary step for fat absorption by the enterocytes.

Designing and synthesizing potent and specific lipase inhibitors is of fundamental value for understanding the mechanisms of lipase action and for various applications, including development of anti-obesity

* Tel.: +30-210-7274462; fax: +30-210-7274761.
E-mail address: gkokotos@cc.uoa.gr (G. Kokotos).

1381-1177/03/$ – see front matter
doi:10.1016/S1381-1177(03)00041-9

agents. In this review, we will discuss results mainly obtained by our group concerning the synthesis of 2-oxo amide triacylglycerol analogues and the inhibition of human pancreatic lipase (HPL) and human gastric lipase (HGL) by 2-oxo amides.

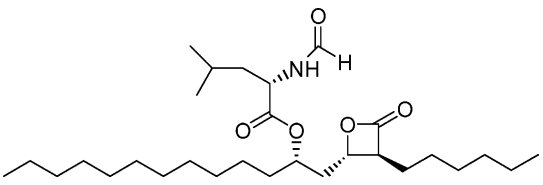
2. Contribution of pancreatic and gastric lipases into fat digestion

In industrially developed countries, the edible lipids present in the human diet are 100 to about 150 g per day, representing 30% of each individual's daily caloric intake. TAGs constitute the major part (95%) of dietary fats. Investigations carried out on healthy volunteers without any pancreatic or gastric deficiency, defined the respective physiological contributions of HPL and HGL in the overall hydrolysis of dietary TAG [11]. It was found that during the digestion of a liquid test meal, 66% of TAG hydrolysis was sufficient for complete absorption and HGL and HPL hydrolyzed 18 and 48% of the TAG-acyl chains, respectively.

Tetrahydrolipstatin (THL, Orlistat®) (Scheme 1) is the hydrogenated derivative of lipstatin, a natural occurring lipase inhibitor, which was isolated from *Streptomyces toxitrocini* in 1987 [12]. THL is the most potent inhibitor of digestive lipases reported up to date. The β-lactone ring of THL reacts with serine 152 of pancreatic lipase to form esters that are slowly hydrolyzed. THL decreases systematic absorption of dietary fat, leading to a reduction in body weight and lowering of plasma cholesterol [13]. In human clinical trials, Orlistat® reduced the absorption of dietary fats by 30%.

3. Lipase inhibitors

Potent and specific lipase inhibitors are of special interest for five reasons:



Scheme 1. Tetrahydrolipstatin.

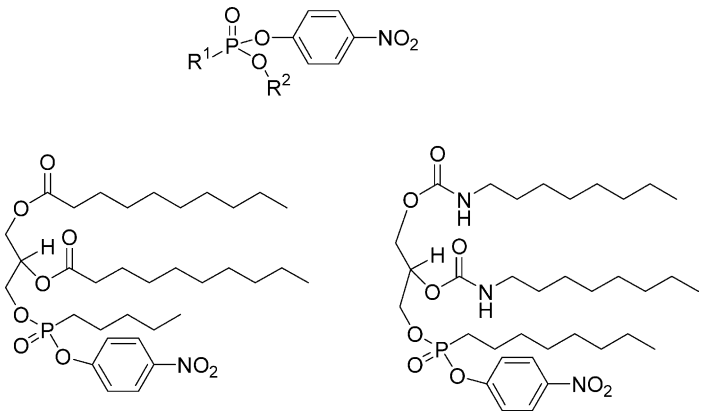
(a) They find applications as anti-obesity agents.
(b) They contribute to a better understanding of the mechanisms of lipase action.
(c) They help to understand the non-catalytic functions of lipases.
(d) They may find applications for the directed molecular evolution of lipolytic enzymes.
(e) They may find applications for the treatment of infectious diseases.

Patkar and Björkling [14] reviewed several families of lipase inhibitors, while the covalent inactivation of lipases was reviewed by Verger and co-workers [15–17]. Among the whole range of lipase inhibitors, two families, β-lactone containing natural products and synthetic alkyl phosphonates, are of special interest. Apart from lipstatin and THL (Scheme 1), some other β-lactone natural products, such as panclicin A–E [18], have been found to inhibit lipase action. Alkyl phosphonates of simple or more complicated structure (some examples are presented in Scheme 2) constitute the major representative class of synthetic lipase inhibitors [17]. They have been found to irreversibly inactivate HPL and HGL as well as microbial lipases. These compounds mimic the transition state that occurs during carboxyester hydrolysis in both their charge distribution and configuration and have been used to solve the 3D structure of various lipases [19].

Experimental studies of the non-catalytic functions of lipoprotein lipase (LPL) require inactivated enzyme [20]. This may be produced by blocking its active site with specific inhibitors like THL or hexadecanesulfonyl fluoride [21]. The inhibition of LPL by alkanesulfonyl fluorides and chlorides has been recently studied [22].

Directed molecular evolution is an attempt to mimic nature by producing enzymes with altered properties (e.g. changes in catalytic properties, stability, specificity or pH profile) [23]. The design, synthesis and inhibition properties of novel inhibitors for directed molecular evolution of lipolytic enzymes by phage-display has been described [24]. The inhibitors are composed of a *p*-nitrophenyl activated phosphonate connected to a biotin moiety through a spacer containing a disulfide bridge.

As has recently been demonstrated, green tea extract (AR25®) inhibits lipolysis of TAGs in gastric



Scheme 2. Phosphonate-type lipase inhibitors.

and duodenal medium in vitro [25]. The natural products berberine and quercetin have been reported to inhibit *Candida rugosa* lipase [26]. The correlation between *Propionibacterium* acne biotypes, lipase activity and rash degree in acne patients has been studied [27]. Kampo formulations, coptidis rhizoma and its alkaloids inhibited lipase activity and suppressed the growth of *Propionibacterium* acnes [28].

4. Rational design of lipase inhibitors

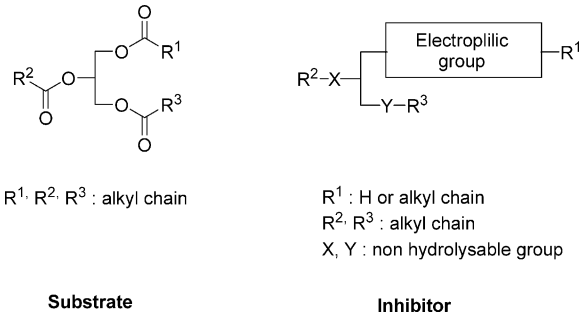
In lipases, the catalytic machinery consists of a triad and an oxyanion hole, which stabilizes the transition state [29]. Both HPL and HGL possess classic catalytic triad (Ser–His–Asp) homologous to that proposed for serine proteases [30], as has been proven by site-directed mutagenesis [31] and crystallographic data [32]. HPL contains an active site with a catalytic triad formed by serine 152, aspartate 176 and histidine 263. The hydrolytic mechanism catalyzed by pancreatic lipase is driven by the nucleophilic attack of serine 152, and the resulting tetrahedral intermediate is stabilized by an oxyanion hole. The acyl enzyme transiently formed is further attacked by a water molecule, thus generating the enzyme and liberating the acyl moiety.

In the case of proteases, progress in drug design has led to the development of small synthetic inhibitors as therapeutic agents [33]. Many inhibitors of serine proteases consist of a substrate-like structure incorporating an activated carbonyl group at the site of the scissile amide bond, capable of forming a covalent intermediate. A number of reactive carbonyl groups, such as fluorinated ketones [34], α-keto esters [35], α-keto amides [36], 1,2-diketones [37], α-keto heterocycles [38] have been successfully used in the design of protease inhibitors. The mechanism of action of these electrophilic inhibitors most likely involves a nucleophilic addition of the active site serine hydroxyl group of the enzyme to the carbonyl group of the inhibitor, with formation of a metastable hemiacetal adduct which mimics the tetrahedral species involved in the enzymatic cleavage of peptide bonds. In the case of some proteases, X-ray [39] and ^{13}C NMR [40] studies have demonstrated the formation of such enzyme-inhibitor hemiacetal adducts.

Synthetic compounds containing electrophilic carbonyl groups, such as fatty alkyl trifluoromethyl ketones [41] and tricarbonyl derivatives of arachidonic and palmitic acids [42], have been reported to inhibit cytosolic phospholipase A_2 ($cPLA_2$) and calcium independent phospholipase A_2 ($iPLA_2$), enzymes containing a Ser residue in their active site [43]. A series of α-keto heterocycles have most recently been reported as exceptionally potent inhibitors of fatty acid amide hydrolase, the enzyme responsible for degradation of endogenous oleamide and anandamide [44].

Taking into consideration the knowledge regarding the mechanism of lipolysis and the structure of serine protease inhibitors, we propose that a lipase inhibitor should consist of two components (Scheme 3):

(a) an electrophilic group that is able to react with the serine residue of the active site;

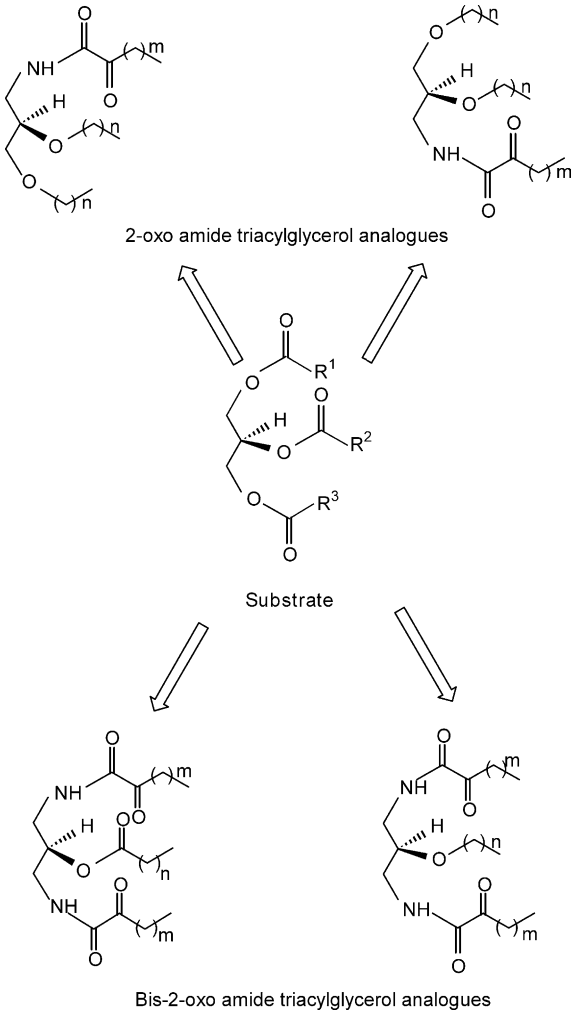


Scheme 3. General structures of lipase substrates and inhibitors.

(b) a lipophilic segment that contains chemical motifs, necessary for both specific interactions and a proper orientation in the enzyme binding pocket.

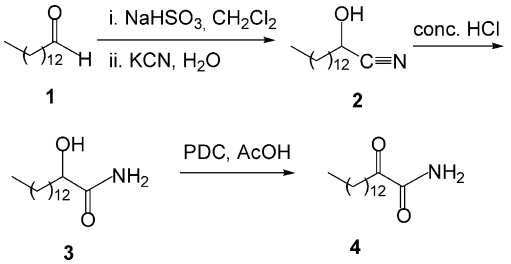
The reactive moiety may be an activated carbonyl group, which is attached to groups enhancing the electrophilicity of the carbonyl. This reactive group must be bound to a lipophilic segment corresponding to the structure of a natural substrate. A glycerol backbone-based structure, containing two or three alkyl or acyl chains, seems ideal for the recognition by the enzyme. The chirality of the molecule and the presence of other functionalities, which may be involved in hydrogen bonding or electrostatic interactions, may enhance the affinity of the inhibitor to a particular lipase.

We chose the 2-oxo amide group as the reactive functionality to replace the scissile ester group of the natural lipase substrate. Ideally, in a triacylglycerol analogue only one 2-oxo amide functionality should be introduced, whereas in the other two positions ester bonds should be maintained. Such a combination of substrate and inhibitor functions in the same molecule complicates the interpretation of the inhibition kinetics. Therefore, the use of non-hydrolyzable ether bonds seems to be unavoidable. Taking into account the structure of TAGs, which are natural lipase substrates, we designed analogues where the ester bond at the *sn*-1 or *sn*-3 position of the substrate was replaced by the 2-oxo amide group (Scheme 4). The two remaining ester bonds were replaced by ether bonds in order to avoid hydrolysis at these positions. Furthermore, the synthesis of 2-oxo amide at the *sn*-1 or *sn*-3 position of triacylglycerol analogues was designed in order to study the stereoselectivity of the inhibition. To ensure the lipophilicity of these analogues, compounds with long alkoxy chains were selected.



Scheme 4. General structures of 2-oxo amide triacylglycerol analogues.

Furthermore, we designed bis-2-oxo amide triacylglycerol analogues, as shown in Scheme 4. The carboxylic ester bonds both at the *sn*-1 and *sn*-3 positions of the substrate were replaced by the 2-oxo amide functionality. The ester bond at the *sn*-2 position was either maintained or replaced by a non-hydrolyzable ether bond. Given the preference of HPL and HGL to hydrolyze ester bonds at the *sn*-1 and *sn*-3 positions, the ester bond at the *sn*-2 position is not anticipated to undergo enzymatic hydrolysis.



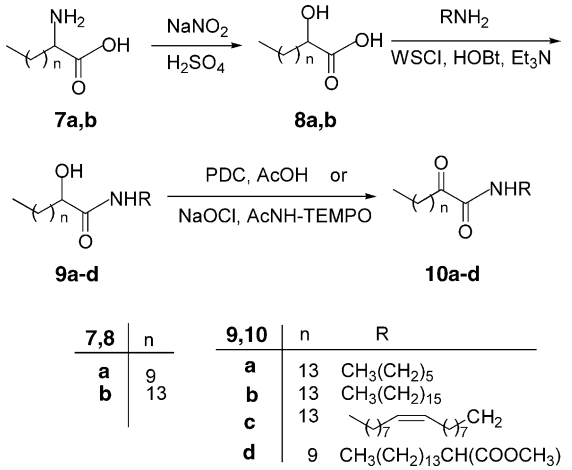
Scheme 5. Synthesis of a primary 2-oxo amide.

5. Synthesis of 2-oxo amides

A number of model primary and *N*-alkyl 2-oxo amides with small, medium or long chains were synthesized following various routes [45]. 2-Oxopentadecanamide (**4**) was prepared from tetradecanaldehyde (**1**) as illustrated in Scheme 5. Addition of KCN to aldehyde **1** afforded cyanohydrin **2**, which was mildly hydrolyzed to 2-hydroxypentadecanamide (**3**). Oxidation of **3** using pyridinium dichromate (PDC) afforded primary 2-oxo amide **4** in high yield.

Commercially available 2-oxo acids can be directly coupled with primary amines by the mixed carbonic anhydride method [46] in high yield. The mixed anhydrides of 2-oxo acids (**5a**,**b**) with isobutyl chloroformate were prepared in situ and were used for coupling with hexylamine, hexadecylamine and *cis*-9-octadecenylamine to afford *N*-alkyl α-keto amides **6a**–**d** (Scheme 6).

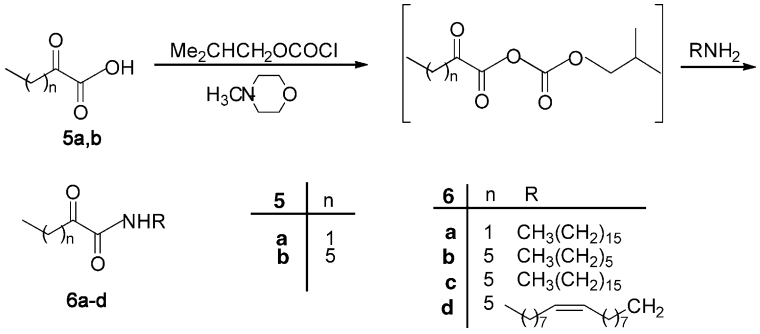
However, long-chain 2-oxo acids are not commercially available. Thus, we developed a general method for the synthesis of *N*-alkyl 2-oxo amides using lipidic α-amino acids as starting material. Lipidic α-amino acids [47] are a class of compounds combining the structural features of amino acids with those of fatty acids. 2-Aminododecanoic acid (**7a**) and 2-aminohexadecanoic acid (**7b**), prepared as described in literature [47], were deaminated by treatment with $NaNO_2$ under acidic conditions (Scheme 7). 2-Hydroxy acids **8a**,**b** were coupled with various amino components using 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (WSCI) [48] as a condensing agent in the presence of 1-hydroxybenzotriazole (HOBt). Apart from linear saturated or unsaturated amines, methyl 2-aminohexadecanoate was used as amino component. The lipophilic *N*-alkyl 2-oxo amides **10a**–**d** were obtained by



7,8	n
a	9
b	13

9,10	n	R
a	13	$CH_3(CH_2)_5$
b	13	$CH_3(CH_2)_{15}$
c	13	$(CH_2)_7CH{=}CH(CH_2)_7CH_2$
d	9	$CH_3(CH_2)_{13}CH(COOCH_3)$

Scheme 7. Synthesis of secondary 2-oxo amides through oxidation of 2-hydroxy amides.



5	n
a	1
b	5

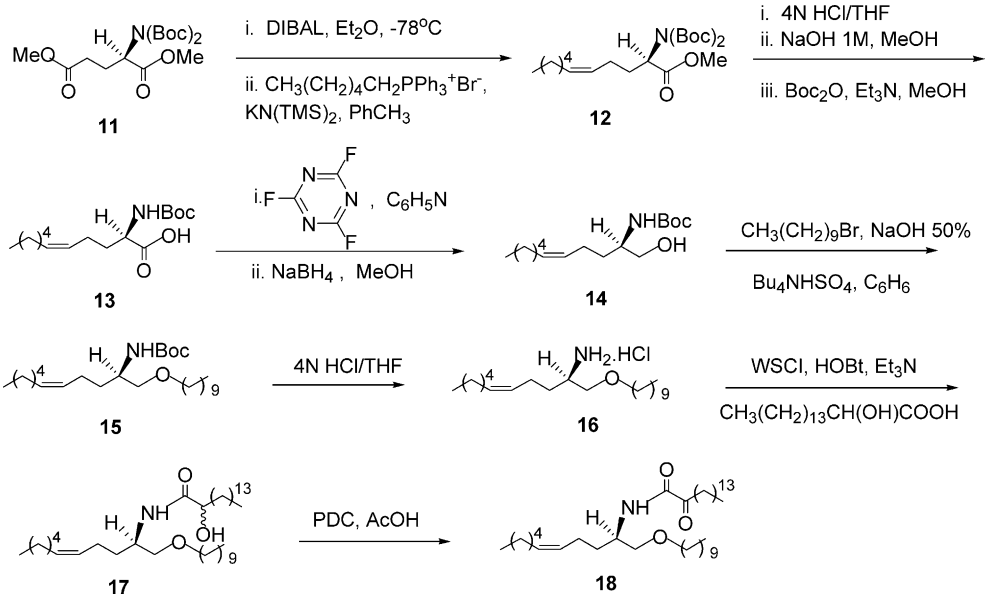
6	n	R
a	1	$CH_3(CH_2)_{15}$
b	5	$CH_3(CH_2)_5$
c	5	$CH_3(CH_2)_{15}$
d	5	$(CH_2)_7CH{=}CH(CH_2)_7CH_2$

Scheme 6. Synthesis of secondary 2-oxo amides through the mixed anhydrides method.

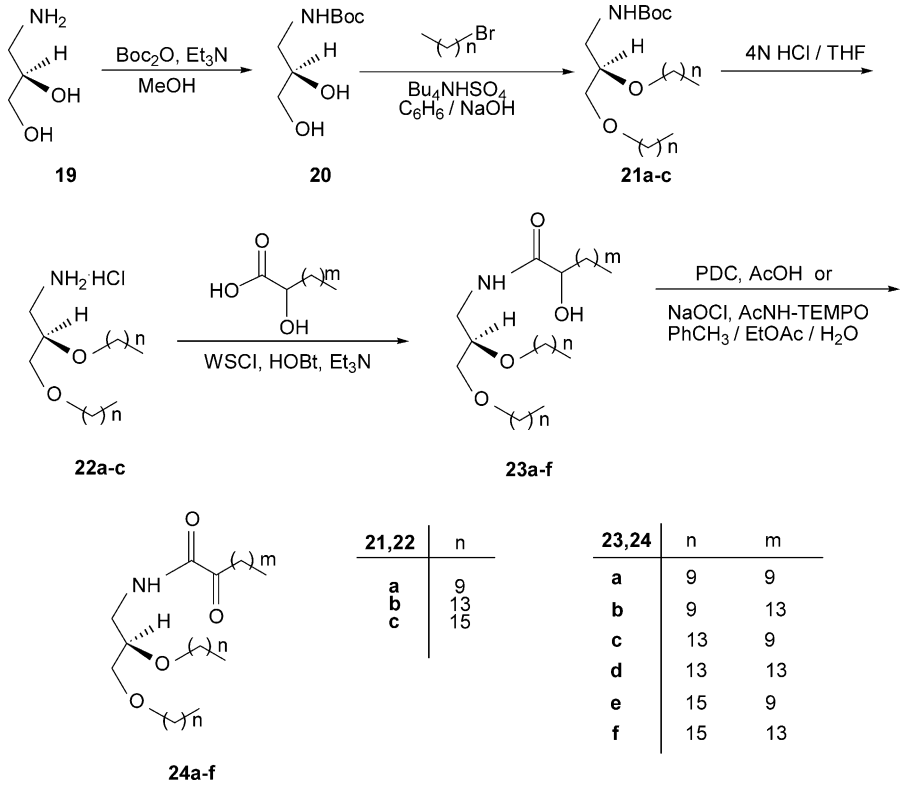
oxidation of **9a**–**d** with either PDC or NaOCl in the presence of catalytic amounts of 4-acetamido-2,2,6,6-tetramethyl-1-piperidinyloxy free radical (AcNH-TEMPO) [49]. In most cases PDC was an effective reagent for the oxidation and afforded the products in good yields. However, the conversion of products with long aliphatic chains proceeded in only moderate yields. In these cases, the use of NaOCl in the presence of AcNH-TEMPO in a biphasic system of toluene, ethyl acetate, dichloromethane and aqueous NaBr proved to be advantageous, giving the oxidized products in almost quantitative yields.

We have also incorporated the 2-oxo amide functionality into a lipidic amino alcohol backbone, thus ensuring the lipophilicity of the final product [50]. The lipidic amino alcohol **14**, used as backbone for the 2-oxo amide inhibitor, was synthesized starting from D-glutamic acid, as described in Scheme 8. Dimethyl *N*,*N*-di-Boc-glutamate (**11**) was reduced using DIBAL under controlled conditions [51] and the resulting aldehyde was submitted to Wittig reaction with the suitable ylide to produce the fully protected unsaturated lipidic amino acid **12**. The Boc-protected amino acid **13** was converted into the corresponding fluoride and reduced in situ [52] by treatment with sodium borohydride and dropwise addition of methanol to produce the amino alcohol **14**. The etherification procedure took place under phase transfer conditions. The hydroxy component **14** was treated with *n*-decylbromide in a biphasic system of benzene/aqueous sodium hydroxide in the presence of catalytic amount of Bu_4NHSO_4 and afforded the ether derivative **15** in satisfactory yield. The Boc group was removed using HCl/THF and the free amino compound **16** was coupled with 2-hydroxyhexadecanoic acid using WSCI/HOBt. The α-hydroxy amide **17** was converted to the corresponding 2-oxo amide **18** using PDC in acetic acid in good yield.

(*S*)- and (*R*)-3-amino-1,2-propanediol (**19**), which are commercially available in optically pure form, were used as starting materials for the preparation of triacylglycerol analogues [53] (Scheme 9, only the (*R*)-enantiomers are shown). The amino group was protected with the *tert*-butoxycarbonyl (Boc) group using di-*tert*-butyldicarbonate (Boc_2O) and triethylamine (Et_3N) [54]. Treatment of the N-protected derivatives **20** with the appropriate alkyl bromide in a biphasic system of benzene/aqueous sodium hydroxide in the presence of catalytic amount of Bu_4NHSO_4 afforded the ether derivatives **21a**–**c** in good yield. Removal of the Boc group using HCl/THF led to the corresponding free amino compounds **22a**–**c**



Scheme 8. Synthesis of an unsaturated lipophilic 2-oxo amide.



21,22	n
a	9
b	13
c	15

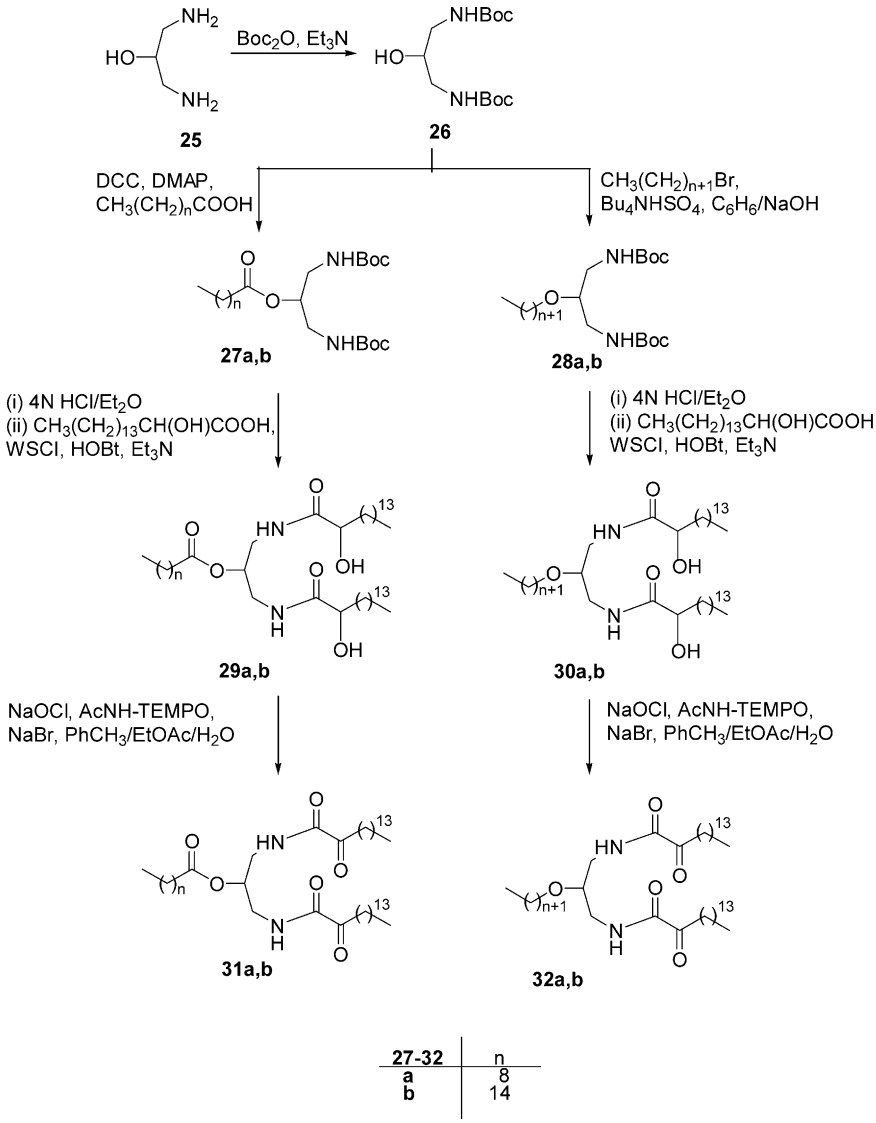
23,24	n	m
a	9	9
b	9	13
c	13	9
d	13	13
e	15	9
f	15	13

Scheme 9. Synthesis of 2-oxo amide triacylglycerol analogues.

and coupling with the suitable 2-hydroxy fatty acid using WSCI/HOBt afforded the 2-hydroxy amides **23a**–**f**. The 2-hydroxy amides were oxidized to the corresponding 2-oxo amides **24a**–**f** using either PDC or NaOCl/AcNH-TEMPO. In the case of 2-hydroxy amides having alkoxy chain lengths of 16 carbons, oxidation to the corresponding 2-oxo amides proceeded only in moderate yield. The use of NaOCl in the presence of catalytic amounts of AcNH-TEMPO, in a biphasic system of toluene, ethyl acetate and aqueous NaBr, not only proved to be very effective for the cases where PDC failed to afford the products in high yield, but also afforded the oxidized products in almost quantitative yield in all the other cases tested. In the case of the substrates with the longer chains, the use of small quantities of CH_2Cl_2 in the solvent system to improve solubility was found to be advantageous.

In the ^{13}C NMR spectra of the 2-oxo amide triacylglycerol analogues, two signals corresponding to carbon atoms of COCONH were clearly assigned [53]. The carbon atom of the 2-oxo group was shifted at 199 ppm due to the presence of the adjacent amide function. The signal of the amide group carbon atom appeared at 160 ppm. In the case of 2-hydroxy amides it appeared at 174 ppm. Furthermore, analysis of the ^{13}C NMR spectra of the 2-hydroxy amides revealed a signal at 72 ppm, which was assigned to the carbon atom of CHOH. In the series of 3-amino-1,2-propanediol derivatives prepared, three signals corresponding to the carbon atoms of 3-amino-1,2-propanediol backbone appeared at 42–40, 77 and 72–70 ppm and were assigned to the CH_2NH, CHO and CH_2O carbon atoms, respectively.

The enantiomeric purities of (*R*)- and (*S*)-**22b** were checked by NMR analysis of their amides with (*R*)-(+)-α-methoxy-α-trifluoromethyl-phenylacetic acid (Mosher acid) [55]. In the ^{19}F NMR spectra of Mosher amides of (*R*)- and (*S*)-**22b**, signals at 8.82 and 8.78 ppm, respectively (using CF_3COOH as an external reference) were observed. Thus, the absence



Scheme 10. Synthesis of bis-2-oxo amide triacylglycerol analogues.

of any diastereomeric fluorine signal indicated enantiomeric excess >95%.

1,3-Diaminopropan-2-ol (**25**) was used as the starting material for the preparation of bis-2-oxo amide analogues [56] (Scheme 10). The amino groups of **25** were protected with the Boc group, and compound **26** was coupled with decanoic and palmitic acid using 1,3-dicyclohexylcarbodiimide (DCC) as a condensing agent in the presence of 4-dimethylaminopyridine (DMAP) [57] to yield compounds **27a**,**b**. The hydroxy component **26** was treated with decyl and hexadecyl bromide and afforded the ether derivatives **28a**,**b**. The Boc groups were removed, and the amino components were coupled with 2-hydroxyhexadecanoic acid using WSCI/HOBt to produce 2-hydroxy amides **29a**,**b** and **30a**,**b**. Bis-2-oxo amides **31a**,**b** and **32a**,**b** were obtained by oxidation of 2-hydroxy amides.

6. Monomolecular film experiments: force/area curves of 2-oxo amides

The use of the monolayer technique, which is based on a decrease in surface pressure due to the film's hydrolysis, is advantageous for the study of lipase inhibition, because conventional emulsified systems do not allow for the control of "interfacial quality" [58]. The monolayer technique is applicable to those enzymatic studies where the lipid film forms a stable monomolecular film at the air/water interface and where the reaction products are freely water soluble and diffuse away rapidly into the aqueous phase [59]. To determine film stability and the interfacial properties of synthetic compounds, force/area curves at the air/water interface should be recorded.

Experiments were performed in the reservoir compartment of a "zero-order" trough. A force/area curve was obtained after spreading a small volume of lipid solution in a volatile solvent (chloroform) at the air/water interface. The surface of the trough was progressively reduced by moving a mobile barrier at a constant rate, and the surface pressure was continuously recorded during compression. Unique features such as area per molecule, collapse pressure, compressibility of the film and possibly phase transition, etc. can be deduced from a force/area curve.

Among the model primary and secondary 2-oxo amides, only derivatives **6d** and **10b**,**c**,**d** were proven to be able to form stable thin films [45]. Recordings of surface pressure dependency as a function of the molecular area of compounds **6d**, **10b**, **10d** and **18** spread over a buffered subphase at pH 8.0 are illustrated in Fig. 1. The large molecular area of the film formed by compound **18** may be attributable to the presence of the double bond as well as that of the two alkyl chains [50].

For compounds **24a–f** the molecular area dependency as a function of the surface pressure of a film spread over a buffered subphase at pH 8.0 is shown in Fig. 2 [53]. The surface pressure/area curves obtained for all pairs of enantiomers were identical. A decrease in the molecular area occupied by the 2-oxo amide compounds was observed as the alkoxy chains increased. This decrease is very clearly illustrated in the case of compounds **24d**,**f** which contain a chain of 13 carbon atoms in the 2-oxo amide moiety. The collapse pressures of compounds **24a**,**b**,**c** are observed at surface pressure values of 20.9, 19.2, 19.8, $mN\,m^{-1}$, respectively and are in the same range as

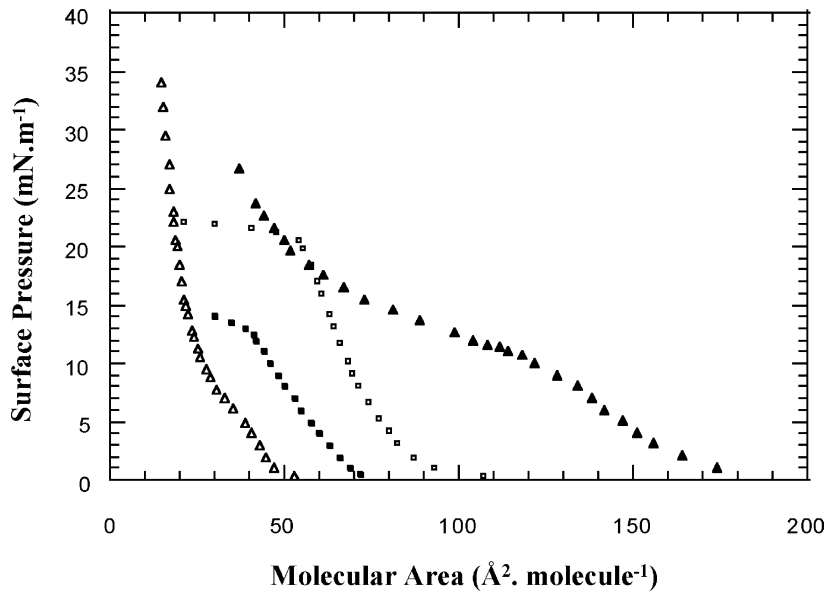


Fig. 1. Force/area curves for compounds **6d** (■), **10b** (△), **10d** (□) and **18** (▲). The aqueous subphase was composed of Tris–HCl 10 mM, pH 8, NaCl 100 mM, $CaCl_2$ 21 mM and EDTA 1 mM. The continuous compression experiment was performed in the rectangular reservoir of the "zero-order" trough.

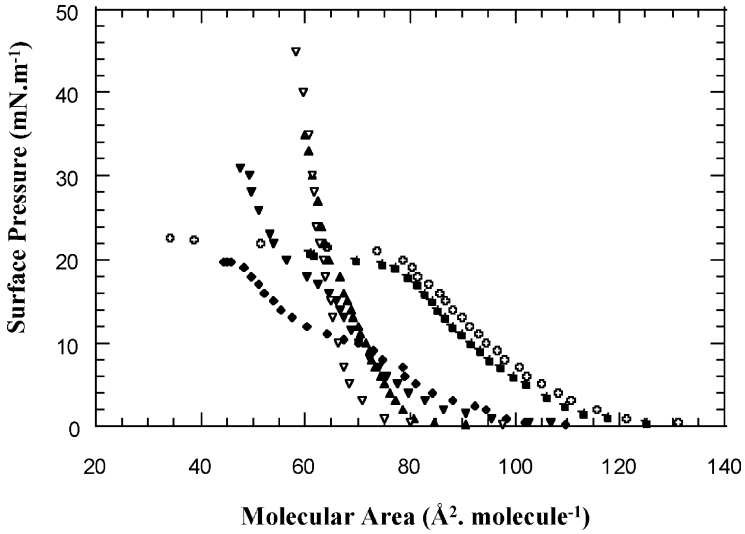


Fig. 2. Force/area curves for compounds **24a** (✪), **24b** (■), **24c** (◆), **24d** (▲), **24e** (▼) and **24f** (▽). The aqueous subphase was composed of Tris–HCl 10 mM, pH 8, NaCl 100 mM, $CaCl_2$ 21 mM and EDTA 1 mM. The continuous compression experiment was performed in the rectangular reservoir of the "zero-order" trough.

the collapse pressure of a trioctanoyl glycerol film. In the case of smaller alkoxy chains, liquid expanded films were obtained while with longer chain lengths liquid condensed (or even solid) force/area curves were recorded. In the case of compounds **24c**,**e**, a clear transition from the liquid expanded to liquid condensed states at around surface pressures of 10 and 18 mN m^{-1}, respectively was observed.

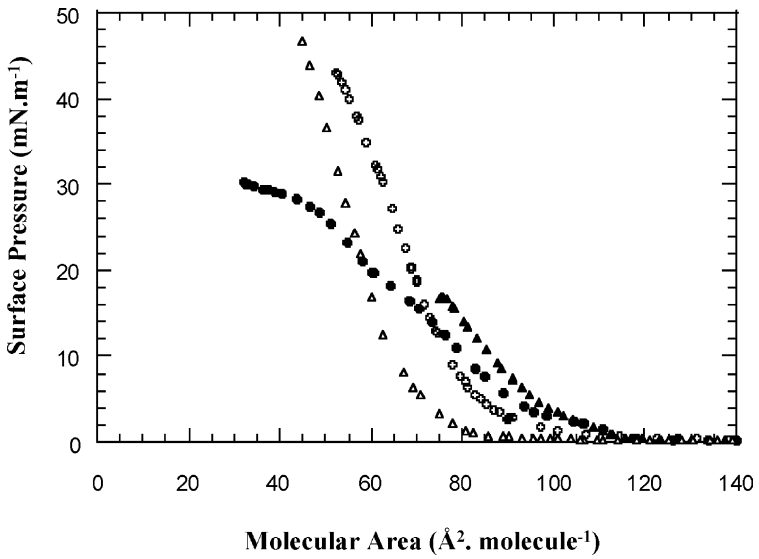


Fig. 3. Force/area curves for compounds **31a** (●), **31b** (△), **32a** (✪) and **32b** (▲). The aqueous subphase was composed of Tris–HCl 10 mM, pH 8, NaCl 100 mM, $CaCl_2$ 21 mM and EDTA 1 mM. The continuous compression experiment was performed in the rectangular reservoir of the "zero-order" trough.

The molecular area dependency for compounds **31a**,**b** and **32a**,**b** as a function of the surface pressure of a film spread over a buffered subphase at pH 8.0 is shown in Fig. 3 [56]. As the alkoxy chains at the *sn*-2 position of compound **31a**,**b** and **32a**,**b** increase, a decrease in the molecular area can be observed.

7. Enzyme kinetics experiments: pancreatic and gastric lipase activity on mixed films containing 2-oxo amide inhibitors

There are at least five major reasons for using lipid monolayers as substrates for lipolytic enzymes [29b]:

(i) It is possible with lipid monolayers to vary and control the "interfacial quality", which depends on the nature of the lipids forming the monolayer, the orientation and conformation of the molecules, the molecular and charge densities, the water structure, the viscosity, etc.
(ii) It is easy to follow the course of the reaction by monitoring one of several physicochemical parameters characteristic of the monolayer film: surface pressure, potential, density, etc.
(iii) Using the surface barostat balance, the lipid packing of a monomolecular film of substrate can be maintained constant during the course of hydrolysis, and it is therefore possible to obtain accurate pre-steady state kinetic measurements with minimal perturbation caused by increasing amounts of reaction products.
(iv) The monolayer technique is highly sensitive and very little lipid is needed to obtain kinetic measurements. This advantage can often be decisive in the case of synthetic or rare lipids.
(v) Inhibition of lipase activity by water-insoluble substrates can be precisely estimated using the "zero-order" trough and mixed monomolecular films in the absence of any synthetic, non-physiological detergent. The monolayer technique is therefore suitable for modeling in vivo situations.

The inhibition experiments were performed using the monolayer technique. For the inhibition studies, the method of "mixed monomolecular films" was used. This method involves the use of a "zero-order" trough, consisting of two compartments: a reaction compartment, where mixed films of substrate and inhibitor are spread, and a reservoir compartment, where only a pure film of substrate is spread. The two compartments are connected to each other by narrow surface channels. When, due to the lipolytic action of the enzyme, the surface pressure decreased, a mobile barrier was moving over the reservoir compartment to compress the film, thus keeping the surface pressure constant.

The inhibition studies for pancreatic lipase with compounds **10d** [45], **18** [50], **24a–f** [53], **31a**,**b** and **32a**,**b** [56] were carried out at 10, 15 or 20 mN m^{-1} depending on the film stability. PPL was active and linear kinetics were recorded at 10, 15 and 20 mN m^{-1}. The inhibitor molar fractions α_{50} obtained for all the 2-oxo amides against pancreatic lipase are summarized in Table 1. The α_{50} value is the inhibitor molar fraction present in 1,2-dicaprin monolayers that causes a 50% decrease in the enzymatic activity. The α_{50} values for **10d** and **18** were found to be 0.100 ± 0.018 and 0.141 ± 0.035, respectively. Although these values correspond to a rather weak inhibition of pancreatic lipase, they are comparable to the α_{50} values recently reported for chiral acylglycerol analogues belonging to the phosphonate-type inhibitors (α_{50} values 0.13–0.20) [60]. No significant differences in the α_{50}

Table 1
Inhibition values of 2-oxo amide and bis-2-oxo amide triacylglycerol analogues on pancreatic lipase with the monolayer technique

Compound	Surface pressure (mN m^{-1})	α_{50}	Reference
10d	20	0.100 ± 0.018[a]	[45]
18	20	0.141 ± 0.009[a]	[50]
(*S*)-**24a**	15	0.052 ± 0.009[a]	[53]
(*S*)-**24b**	15	0.050 ± 0.009[a]	[53]
(*R*)-**24b**	15	0.062 ± 0.008[a]	[53]
(*S*)-**24c**	10	0.046 ± 0.005[a]	[53]
(*R*)-**24c**	10	0.070 ± 0.008[a]	[53]
(*S*)-**24d**	15	0.056 ± 0.007[a]	[53]
(*R*)-**24d**	15	0.053 ± 0.005[a]	[53]
(*S*)-**24e**	10	0.050 ± 0.007[a]	[53]
(*R*)-**24e**	10	0.045 ± 0.004[a]	[53]
(*S*)-**24f**	15	0.045 ± 0.004[a]	[53]
(*R*)-**24f**	15	0.044 ± 0.006[a]	[53]
31a	15	0.076 ± 0.004[b]	[56]
31b	15	0.173 ± 0.036[b]	[56]
32a	15	0.134 ± 0.015[b]	[56]
32b	15	0.240 ± 0.085[b]	[56]

[a] PPL.
[b] HPL.

Table 2
Inhibition values of 2-oxo amide and bis-2-oxo amide triacylglycerol analogues on human gastric lipase with the monolayer technique

Compound	Surface pressure ($mN\,m^{-1}$)	α_{50}	Reference
(*R*)-**24b**	15	0.079 ± 0.018	[53]
(*S*)-**24b**	15	0.159 ± 0.058	[53]
(*R*)-**24d**	15	0.056 ± 0.008	[53]
(*S*)-**24d**	15	0.108 ± 0.032	[53]
(*R*)-**24f**	15	0.061 ± 0.015	[53]
(*S*)-**24f**	15	0.141 ± 0.051	[53]
31a	20	0.020 ± 0.004	[56]
31b	20	0.045 ± 0.009	[56]
32a	20	0.109 ± 0.011	[56]
32b	20	0.171 ± 0.019	[56]

values were observed when the chain length of either the ether or the 2-oxo amide moieties of **24a–f** was varied. In the case of PPL, the configuration at the *sn*-2 carbon did not affect the α_{50} value. This behaviour may be attributed to the low stereopreference of PPL [61], in agreement with the data obtained with HPL against TAGs and triacylglycerol analogues [61,62].

The inhibitor molar fractions α_{50} obtained for all the triacylglycerol analogues **24b**,**d**,**f** [53], **31a**,**b** and **32a**,**b** [56] against HGL are summarized in Table 2. HGL was active at 15 and $20\,mN\,m^{-1}$ and linear kinetics were recorded. Contrary to the results obtained with PPL for the chiral 2-oxo amides **24a–f**, in the case of HGL the three pairs of enantiomers of 2-oxo amide triacylglycerol analogues tested, (*R*)- and (*S*)-**24b**,**d**,**f**, displayed differential inhibitory effects. The inhibition depends on the configuration at the *sn*-2 carbon of the glycerol backbone. The enantiomers with the (*R*)-configuration proved to be better (two-fold) inhibitors than the corresponding structures with the (*S*)-configuration [53]. Furthermore, the results obtained indicate a dependency of the α_{50} value upon the length of the alkoxy chain. A minimum in the α_{50} value, 0.056 ± 0.009, for compound (*R*)-**24d** was observed with a chain length of fourteen carbon atoms.

As can be seen from the data obtained for bis-2-oxo amides, the length of the *sn*-2 chain (acyloxy or alkoxy) influences the potency of the inhibition. A chain length decrease from 16 (**31b** and **32b**) to 10 (**31a** and **32a**) carbon atoms causes approximately a two-fold decrease of the α_{50} values determined for both HGL and HPL [56]. HGL seems to have a strong preference for the ester derivatives of the bis-2-oxo amide triacylglycerol analogues **31a**,**b** as compared to the corresponding ether derivatives **32a**,**b** [56]. The ester derivatives **31a**,**b** are four- to five-fold more potent than the corresponding inhibitors **32a**,**b** containing ether bonds. This observation suggests that the carbonyl oxygen is important for inhibition, probably via the existence of specific interactions with HGL. It is relevant to note that the nature of the chemical bond at the *sn*-2 position of substrates influences the stereoselectivity of various microbial lipases [63], while the presence of amide or carbamoyl groups at the *sn*-1 and *sn*-2 positions of inhibitors enhances the potency of *Staphylococcus hyicus* lipase inhibition by α-keto triglyceride analogues [64].

Among all the 2-oxo amide derivatives, compound **31a** proved to be the most potent HGL inhibitor, causing a 50% decrease at 0.020 surface molar fractions. Currently, the best synthetic HPL inhibitor reported in the literature is *O*-hexadecyl-*O*-(*p*-nitrophenyl) *n*-undecyl phosphonate, with an α_{50} value of 0.003 [65]. In the case of HGL, the highest inhibition was obtained with *O*-undecyl-*O*-(*p*-nitrophenyl) *n*-decyl phosphonate, which exhibits an α_{50} value of 0.008 [65]. Thus, bis-2-oxo amide **31a** displays an inhibitory effect of the same order of magnitude with the most potent synthetic HGL inhibitor. Tetrahydrolipstatin exhibits an α_{50} value of 0.0025 for HGL [66]; this registered anti-obesity drug is 10-fold more potent than 1,3-bis-2-oxo amide **31a**.

8. Conclusions

Our results indicate that the 2-oxo amide group is a valuable substituent for the future design and synthesis of powerful lipolytic enzyme inhibitors. Mono 2-oxo amide triacylglycerol analogues displayed higher inhibitory effects on HPL than on HGL, whereas bis-2-oxo amide triacylglycerol analogues containing an ester group at the *sn*-2 position are potent inhibitors of HGL. The proposed strategy for rational design of lipase inhibitors may also be applied to other lipolytic enzymes. Thus, novel potent and specific inhibitors

of lipolytic enzymes with industrial and biomedical interest are expected to be developed in the near future.

Acknowledgements

I wish to thank the following members of my research group: Dr. A. Chiou, Dr. T. Markidis, Dr. S. Kotsovolou, V. Magrioti for constructive discussion in preparing this review. I am indebted to Dr. R. Verger (Laboratoire de Lipolyse Enzymatique, CNRS Marseille) and Dr. V. Constantinou-Kokotou (Agricultural University of Athens) for fruitful discussions.

References

[1] (a) B. Borgström, H.L. Brockman, Lipases, Elsevier, Amsterdam, 1984;
(b) P. Wooley, S.B. Petersen, Lipases: Their Structure, Biochemistry and Applications, Cambridge University Press, Cambridge, 1994.
[2] K.D. Mukherjee, M.J. Hills, in: P. Wooley, S.B Petersen (Eds.), Lipases: Their Structure, Biochemistry and Application, Cambridge University Press, Cambridge, 1994, p. 49.
[3] M. Iwai, Y. Tsujisaka, in: B. Borgström, H.L. Brockman (Eds.), Lipases, Elsevier, Amsterdam, 1984, p. 443.
[4] (a) K.-E. Jaeger, S. Ransac, B.W. Dijkstra, C. Colson, M. Vanheuvel, O. Misset, FEMS Microbiol. Rev. 15 (1994) 29;
(b) K.-E. Jaeger, M. Reetz, Trends Biotechnol. 16 (1998) 396.
[5] L. Sarda, P. Desnuelle, Biochim. Biophys. Acta 30 (1958) 513.
[6] (a) D.L. Ollis, E. Cheah, M. Cygler, B. Dijkstra, F. Frolow, S.M. Franken, M. Harel, S.J. Remington, I. Silman, J. Schrag, J.L. Sussman, K.H.G. Verschueren, A. Goldman, Protein Eng. 5 (1992) 197;
(b) M. Cygler, J. Schrag, J.L. Sussman, M. Harel, I. Silman, M.K. Gentry, B.P. Doctor, Protein Sci. 2 (1993) 366.
[7] R. Verger, Trends Biotechnol. 15 (1997) 32.
[8] (a) K. Faber, Biotransformations in Organic Chemistry, Springer, Berlin, 1995;
(b) U.T. Bornscheuer, R.J. Kazlauskas, Hydrolases in Organic Synthesis, VCH, Weinheim, 1999.
[9] (a) A.M. Klibanov, Trends Biotechnol. 15 (1997) 97;
(b) R.D. Schmid, R. Verger, Angew. Chem. Int. Ed. 37 (1998) 1608;
(c) M.T. Reetz, K.-E. Jaeger, Top. Curr. Chem. 200 (1999) 31;
(d) Y.L. Khmelnitsky, J.O. Rich, Curr. Opin. Chem. Biol. 3 (1999) 47;
(e) J. Pleiss, M. Fischer, M. Peiker, C. Thiele, R.D. Schmid, J. Mol. Catal. B: Enzym. 10 (2000) 491;
(f) P. Berglund, Biomol. Eng. 18 (2001) 13.
[10] (a) R. Verger, M. Aoubala, F. Carriere, S. Ransac, L. Dupuis, J. de Caro, F. Ferrato, I. Douchet, R. Laugier, A. de Caro, Proc. Nutr. Soc. 55 (1996) 5;
(b) M.E. Lowe, Gastroenterology 107 (1994) 1524.
[11] F. Carriere, J.A. Barrowman, R. Verger, R. Laugier, Gastroenterology 105 (1993) 876.
[12] (a) E.K. Weibel, P. Hadvary, E. Hochuli, E. Kupfer, H. Langsfeld, J. Antibiot. 40 (1987) 1081;
(b) E. Hochuli, E. Kupfer, R. Maurer, W. Meister, Y. Mercadel, K. Schmidt, J. Antibiot. 40 (1987) 1086.
[13] (a) J.B. Hauptman, F.S. Jeunet, D. Hartmann, Am. J. Clin. Nutr. 55 (1992) 309;
(b) M.L. Drent, E.A. Vanderveen, Int. J. Obes. 17 (1993) 241;
(c) M.L. Drent, I. Larsson, T. William-Olson, F. Quaade, F. Czubayko, K. von Bergmann, W. Strobel, L. Sjöström, E.A. van der Veen, Int. J. Obes. 19 (1995) 221;
(d) N. Finer, W.P.T. James, P.G. Kopelman, M.E.J. Lean, G. Williams, Int. J. Obes. 24 (2000) 306.
[14] S. Patkar, F. Björkling, in: P. Wooley, S.B. Petersen (Eds.), Lipases: Their Structure, Biochemistry and Application, Cambridge University Press, Cambridge, 1994, p. 207.
[15] S. Ransac, Y. Gargouri, F. Marguet, G. Buono, C. Beglinger, P. Hildebrand, H. Lengsfeld, P. Hadvary, R. Verger, Methods Enzymol. 286 (1997) 190.
[16] Y. Gargouri, S. Ransac, R. Verger, Biochim. Biophys. Acta 1344 (1997) 6.
[17] J.-F. Cavalier, G. Buono, R. Verger, Acc. Chem. Res. 33 (2000) 579.
[18] (a) K. Yoshinari, N. Aoki, T. Ohtsuka, N. Nakayama, Y. Itezono, M. Mutoh, J. Watanabe, K. Yokose, J. Antibiot. 47 (1994) 1376;
(b) M. Mutoh, N. Nakada, S. Matsukuma, S. Ohshima, K. Yoshinari, J. Watanabe, M. Arisawa, J. Antibiot. 47 (1994) 1369.
[19] (a) M. Cygler, P. Grochulsi, R.J. Kazlauskas, R.D. Schrag, F. Bouthillier, B. Rubin, A.N. Serreqi, A.K. Gupta, J. Am. Chem. Soc. 116 (1994) 3180;
(b) M.P. Egloff, F. Marguet, G. Buono, R. Verger, C. Cambillau, H. van Tilbeurgh, Biochemistry 34 (1995) 2751;
(c) S. Longhi, M. Mannesse, H.M. Verheij, G.H. de Hass, M. Egmond, E. Knoops-Mouthuy, C. Cambillau, Protein Sci. 6 (1997) 275;
(d) A. Roussel, N. Miled, L. Berti-Dupuis, M. Riviere, S. Spinelli, P. Berna, V. Gruber, R. Verger, C. Cambillau, J. Biol. Chem. 277 (2002) 2266.
[20] G. Olivecrona, A. Lookene, Methods Enzymol. 286 (1997) 102.
[21] (a) A. Lookene, N. Scottova, G. Olivecrona, Eur. J. Biochem. 222 (1994) 395;
(b) N. Skottova, R. Savonen, A. Lookene, M. Hultin, G. Olivecrona, J. Lipid Res. 36 (1995) 1334.
[22] G. Kokotos, S. Kotsovolou, V. Constantinou-Kokotou, G. Wu, G. Olivecrona, Bioorg. Med. Chem. Lett. 10 (2000) 2803.
[23] (a) K.-E. Jaeger, M.T. Reetz, Curr. Opin. Chem. Biol. 4 (2000) 68;

(b) M.T. Reetz, K.-E. Jeager, Chem. Eur. J. 6 (2000) 407;
(c) M.T. Reetz, Angew. Chem. Int. Ed. Engl. 40 (2001) 285;
(d) U.T. Bornscheuer, M. Pohl, Curr. Opin. Chem. Biol. 5 (2001) 137.
[24] (a) H.-J. Deussen, S. Danielsen, J. Breinholt, T.V. Borchert, Bioorg. Med. Chem. 8 (2000) 507;
(b) H.-J. Deussen, S. Danielsen, J. Breinholt, T.V. Borchert, Bioorg. Med. Chem. Lett. 10 (2000) 2027.
[25] C. Juhel, M. Armand, Y. Pafumi, C. Rosier, J. Vandermander, D. Lairon, J. Nutr. Biochem. 11 (2000) 45.
[26] (a) E. Grippa, R. Valla, L. Battinelli, G. Mazzanti, L. Saso, B. Silvestrini, Biosci. Biotechnol. Biochem. 63 (1999) 1557;
(b) M.-T. Gatto, S. Falcocchio, E. Grippa, G. Mazzanti, L. Battinelli, G. Nicolosi, D. Lambusta, L. Saso, Bioorg. Med. Chem. 10 (2002) 269.
[27] S. Higaki, T. Kitagawa, M. Kagoura, M. Morohashi, T. Yamagishi, J. Dermatol. 27 (2000) 519.
[28] S. Higaki, M. Nakamura, M. Morohashi, Y. Hasegawa, T. Yamagishi, J. Dermatol. 23 (1996) 310.
[29] (a) M.E. Lowe, in: C.M. Mansbach (Ed.), Intestinal Lipid Metabolism, Kluwer Academic Publishers, Dordrecht, 2001, p. 37;
(b) S. Ransac, F. Carrière, E. Rogalska, R. Verger, F. Marguet, G. Buono, E.P. Melo, J.M.S. Cabral, M.-P.E. Egloff, H. van Tilbeurgh, C. Cambillau, NATO ASI Ser. H96 (1996) 265.
[30] D. Blow, Nature 351 (1991) 444.
[31] M.E. Lowe, J. Biol. Chem. 267 (1992) 17069.
[32] (a) F.K. Winkler, A. D'Arcy, W. Hunziker, Nature 343 (1990) 771;
(b) L. Brady, A.M. Brzozowski, Z.S. Derewenda, E. Dodson, G. Dodson, S. Tolley, J.P. Turkenburg, L. Christiansen, B. Hughe-Jensen, L. Norskov, L. Thim, U. Menge, Nature 343 (1990) 767;
(c) H. van Tilbeurgh, M.-P. Egloff, C. Martinez, N. Rugani, R. Verger, C. Cambillau, Nature 362 (1993) 814;
(d) A. Roussel, S. Canaan, M.-P. Egloff, M. Riviere, L. Dupuis, R. Verger, C. Cambillau, J. Biol. Chem. 274 (1999) 16995.
[33] D. Leung, G. Abbenante, D.P. Fairlie, J. Med. Chem. 43 (2000) 305.
[34] (a) B. Imperiali, R.H. Abeles, Biochemistry 25 (1986) 3760;
(b) J.-P. Beugue, D. Bonnet-Delpon, Tetrahedron 47 (1991) 3207.
[35] Z. Li, G.S. Patil, Z.E. Colubski, H. Hori, K. Tehrani, J.E. Foreman, D.D. Eveleth, R.T. Bartus, J.C. Powers, J. Med. Chem. 36 (1993) 3472.
[36] Z. Li, A.-C. Ortega-Vilain, G.S. Patil, D.-L. Chu, J.E. Foreman, D.D. Eveleth, J.C. Powers, J. Med. Chem. 39 (1996) 4089.
[37] S. Mehdi, M.R. Angelastro, J.P. Burkhart, J.R. Kehl, N.P. Peet, P. Bey, Biochem. Biophys. Res. Commun. 166 (1990) 595.
[38] S. Tsutsumi, T. Okonogi, S. Shibahara, S. Ohuchi, E. Hatsushiba, A.A. Patchett, G. Christensen, J. Med. Chem. 37 (1994) 3492.
[39] (a) P.D. Edwards, E.F. Meyer, J.J. Vijayalakshmi, P.A. Tuthill, D.A. Andisik, G. Gomes, A. Strimpler, J. Am. Chem. Soc. 114 (1992) 1854;
(b) A.Y. Lee, M. Hagihara, R. Karmacharya, M.W. Albers, S.L. Schreiber, J. Clardy, J. Am. Chem. Soc. 115 (1993) 12619;
(c) K. Hakansson, A. Tulinsky, M.M. Abelman, T.A. Miller, G.P. Vlasuk, P.W. Bergum, M.S.L. Lim-Wilby, T.K. Brunck, Bioorg. Med. Chem. 3 (1995) 1009.
[40] T.-C. Liang, R.H. Abeles, Biochemistry 26 (1987) 7603.
[41] (a) F. Ghomaschi, R. Loo, J. Balsinde, F. Bartoli, R. Apitz-Castro, J. Clark, E.A. Dennis, M.H. Gelb, Biochim. Biophys. Acta 1420 (1999) 45;
(b) E.J. Ackerman, K. Conde-Frieboes, E.A. Dennis, J. Biol. Chem. 270 (1995) 445;
(c) I.P. Street, H.-K. Lin, F. Laliberte, F. Ghomashchi, Z. Wang, H. Perrier, N.M. Tremblay, N.M. Huang, P.K. Weech, M.H. Gelb, Biochemistry 32 (1993) 5935.
[42] (a) K.C. Frieboes, L.J. Reynolds, Y.C. Lio, M.R. Hale, H.H. Wasserman, E.A. Dennis, J. Am. Chem. Soc. 118 (1996) 5519;
(b) H.H. Wasserman, D.S. Ennis, P.L. Power, M.J. Ross, J. Org. Chem. 58 (1993) 4785.
[43] D.A. Six, E.A. Dennis, Biochim. Biophys. Acta 1488 (2000) 1.
[44] D.L. Boger, H. Sato, A.E. Lerner, M.P. Hadrick, R.A. Fecik, H. Miyauchi, G.D. Wilkie, B.J. Austin, M.P. Patricelli, B.F. Cravatt, Proc. Natl. Acad. Sci. U.S.A. 97 (2000) 5044.
[45] A. Chiou, R. Verger, G. Kokotos, Lipids 36 (2001) 535.
[46] G.W. Anderson, J.E. Zimmerman, F.M. Callahan, J. Am. Chem. Soc. 89 (1967) 5012.
[47] (a) G. Kokotos, V. Martin, V. Constantinou-Kokotou, W.A. Gibbons, Amino Acids 11 (1996) 329;
(b) V. Constantinou, G. Kokotos, Amino Acids 16 (1999) 237.
[48] J.C. Sheehan, P.A. Cruickshank, G.L. Boshart, J. Org. Chem. 26 (1961) 2525.
[49] (a) M.R. Leanna, T.J. Sowin, H.E. Morton, Tetrahedron Lett. 33 (1992) 5029;
(b) Z. Ma, J.M. Bobbitt, J. Org. Chem. 56 (1991) 6110.
[50] A. Chiou, T. Markidis, V. Constantinou-Kokotou, R. Verger, G. Kokotos, Org. Lett. 2 (2000) 347.
[51] G. Kokotos, J.-M. Padron, T. Martin, W.A. Gibbons, V.S. Martin, J. Org. Chem. 63 (1998) 3741.
[52] G. Kokotos, C. Noula, J. Org. Chem. 61 (1996) 6994.
[53] G. Kokotos, R. Verger, A. Chiou, Chem. Eur. J. 6 (2000) 4211.
[54] E. Ponnusamy, U. Fotadar, A. Spisni, D. Flat, Synthesis (1986) 48.
[55] G.A. Dale, D.L. Dull, H.S. Mosher, J. Org. Chem. 34 (1969) 2543.
[56] S. Kotsovolou, A. Chiou, R. Verger, G. Kokotos, J. Org. Chem. 66 (2001) 962.
[57] B. Neises, W. Steglich, Angew. Chem. Int. Ed. Engl. 17 (1978) 522.
[58] R. Verger, G.H. de Haas, Annu. Rev. Biophys. Bioeng. 5 (1976) 77.
[59] S. Ransac, M.G. Ivanova, R. Verger, I. Panaiotov, Methods Enzymol. 286 (1997) 263.
[60] F. Marguet, I. Douchet, J.-F. Cavalier, G. Buono, R. Verger, Colloids Surf. B 13 (1999) 37.

[61] E. Rogalska, C. Cudrey, F. Ferrato, R. Verger, Chirality 5 (1993) 24.
[62] S. Ransac, E. Rogalska, Y. Gargouri, A.M.T.J. Deveer, F. Paltauf, G.H. de Haas, R. Verger, J. Biol. Chem. 265 (1990) 20263.
[63] P. Stadler, A. Kovac, L. Haalck, F. Spener, F. Paltauf, Eur. J. Biochem. 227 (1995) 335.
[64] J.-W.F.A. Simons, R.C. Cox, M.R. Egmond, H.M. Verjeij, Biochemistry 38 (1999) 6346.
[65] J.-F. Cavalier, S. Ransac, R. Verger, G. Buono, Chem. Phys. Lipids 100 (1999) 3.
[66] S. Ransak, Y. Gargouri, H. Moreu, R. Verger, Eur. J. Biochem. 202 (1991) 395.