Bis-2-oxo Amide Triacylglycerol Analogues: A Novel Class of Potent Human Gastric Lipase Inhibitors

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A novel class of potent human gastric lipase inhibitors, bis-2-oxo amide triacylglycerol analogues, was developed. These analogues of the natural substrate of lipases were prepared starting from 1,3-diaminopropan-2-ol. They were designed to contain the 2-oxo amide functionality in place of the scissile ester bond at the *sn*-1 and *sn*-3 position, while the ester bond at the *sn*-2 position was either maintained or replaced by an ether bond. The derivatives synthesized were tested for their ability to form stable monomolecular films at the air/water interface by recording their surface pressure/molecular area compression isotherms. The inhibition of human pancreatic and gastric lipases by the bis-2-oxo amides was studied using the monolayer technique with mixed films of 1,2-dicaprin containing variable proportions of each inhibitor. The nature of the functional group (ester or ether), as well as the chain length, at the *sn*-2 position influenced the potency of the inhibition. Among the compounds tested, 2-[(2-oxohexadecanoyl)amino]-1-[[(2-oxohexadecanoyl)-amino]methyl]ethyl decanoate was the most potent inhibitor, causing a 50% decrease in HPL and HGL activities at 0.076 and 0.020 surface molar fractions, respectively.

Introduction

The hydrolysis of dietary triacylglycerols by digestive lipases (pancreatic and gastric) to monoacylglycerols and free fatty acids is a necessary step for efficient fat digestion and absorption by the enterocytes.[1] Potent and specific inhibitors of lipases are of interest because they may find applications as anti-obesity agents.[2] The β-lactone-containing inhibitor tetrahydrolipstatin is already in clinical use for the treatment of obesity.[3] Lipase inhibitors may also contribute to a better understanding of the mechanisms of lipase action.[4] In lipases, the catalytic machinery consists of a triad and an oxyanion hole, which stabilizes the transition state. Both human pancreatic (HPL) and human gastric lipase (HGL) possess a classical catalytic triad (Ser-His-Asp) homologous to that proposed for serine esterases.[5]

Synthetic compounds containing electrophilic carbonyl groups, such as fatty alkyl trifluoromethyl ketones[6] and tricarbonyl derivatives of arachidonic and palmitic acids,[7] 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.[8] Most recently, we have reported that lipophilic α-keto amides inhibit pancreatic lipase.[9] Furthermore, α-keto triglyceride analogues were shown to inhibit *Staphylococcus hyicus* lipase.[10] A series of α-keto hetero-

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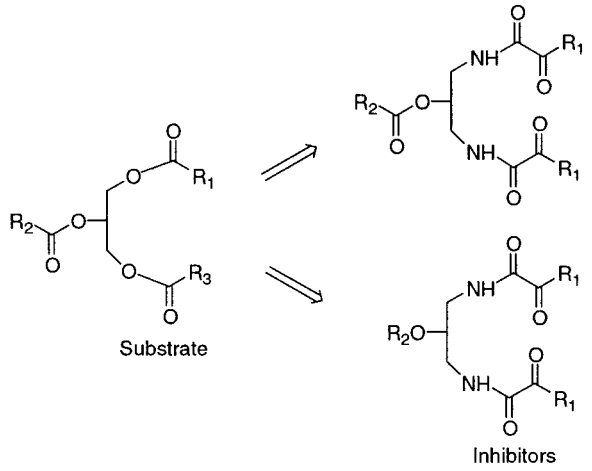
Scheme 1. Structures of Inhibitors in Comparison with Triacylglycerols, the Natural Substrate of Lipases



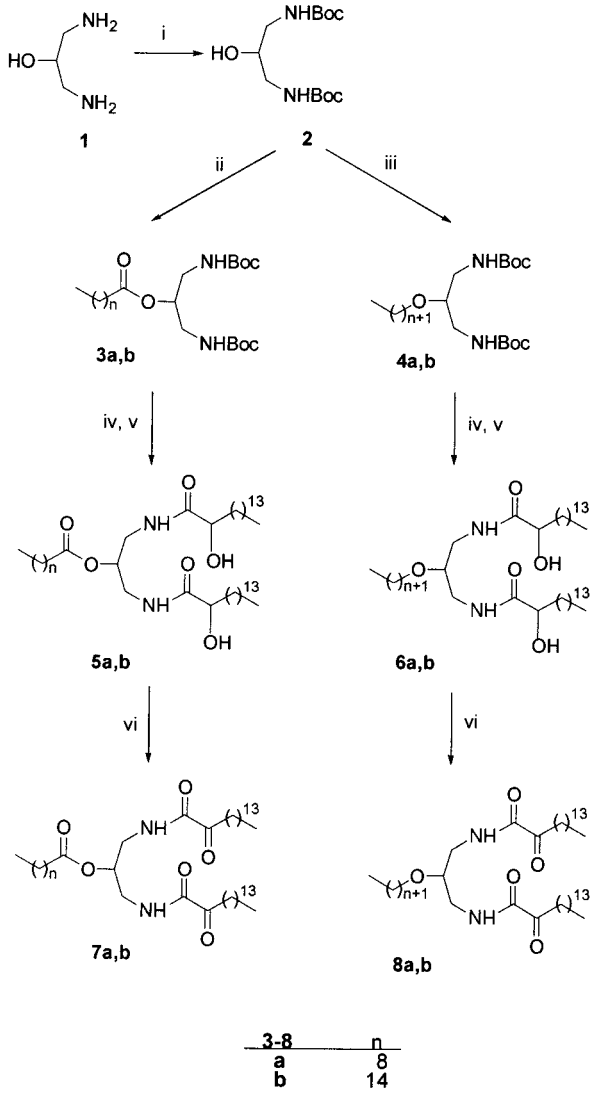
cycles have most recently been reported as exceptionally potent inhibitors of fatty acid amide hydrolase, the enzyme responsible for degradation of endogenous oleamide and anandamide.[11] To extend our studies on the development of novel inhibitors of digestive lipases, we report here the synthesis of bis-2-oxo amide triacylglycerol analogues, the study of their surface properties, and their inhibitory effect on HPL and HGL activities studied by the monomolecular film technique.

Results and Discusssion

In general, an enzyme inhibitor consists of two components: a chemically reactive moiety, capable of reacting with the catalytic site of the enzyme and a part that contains chemical motifs, necessary for specific interactions and a proper orientation in the enzyme binding pocket. The novel 2-oxo amides were designed taking into consideration the chemical structure of triacylgycerols, which are the natural substrate of lipases (Scheme 1). 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.

1,3-Diaminopropan-2-ol (**1**) was used as starting material for the preparation of the target compounds. The amino groups of **1** were protected with the *tert*-butoxycarbonyl (Boc) group using di-*tert*-butyl dicarbonate (Boc_2O) and triethylamine.[12] Compound **2** was coupled with decanoic and palmitic acid using 1,3-dicyclohexylcarbodiimide (DCC) as a condensing agent in the presence of 4-(dimethylamino)pyridine (DMAP)[13] to produce compounds **3a**,**b** (Scheme 2). The etherification procedure took place under phase-transfer conditions. The hydroxy

Scheme 2. Synthesis of Bis-2-oxo Amide Triacylglycerol Analogues[a]



[a] Reagents and conditions: (i) Boc_2O, Et_3N; (ii) DCC, DMAP, $CH_3(CH_2)_nCOOH$; (iii) $CH_3(CH_2)_{n+1}Br$, Bu_4NHSO_4, C_6H_6/NaOH; (iv) 4 N HCl/Et_2O; (v) $CH_3(CH_2)_{13}CH(OH)COOH$, WSCI, HOBt, Et_3N; (vi) AcNH−TEMPO, NaBr, NaOCl, $PhCH_3$, EtOAc, H_2O.

component **2** was treated with decyl and hexadecyl bromide in a biphasic system of benzene/aqueous sodium hydroxide in the presence of a catalytic amount of Bu_4NHSO_4 and afforded the ether derivatives **4a**,**b**. Removal of the Boc groups with HCl/Et_2O led to the corresponding free amino compounds, which were coupled with 2-hydroxyhexadecanoic acid using 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (WSCI)[14] as a condensing agent in the presence of 1-hydroxybenzotriazole (HOBt) to produce 2-hydroxy amides **5a**,**b** and **6a**,**b**. The racemic 2-hydroxy fatty acids were prepared by deamination of the corresponding 2-amino fatty acids[15] using $NaNO_2$ under acidic conditions. The 2-hydroxy amides were oxidized to the corresponding 2-oxo amides **7a**,**b** and **8a**,**b** using either pyridinium dichromate (PDC) or NaOCl in the presence of 4-acetamido-2,2,6,6-tetramethyl-1-piperidinyloxy free radical (AcNH−TEMPO).[16]

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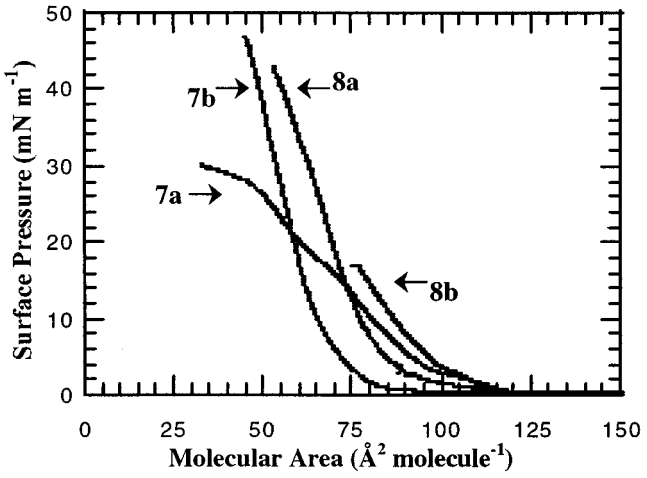

Figure 1. Force/area curves for compounds **7a**,**b** and **8a**,**b**. The aqueous subphase was composed of Tris/HCl 10 mM, pH 8, NaCl 150 mM, $CaCl_2$ 21 mM, EDTA 1 mM. The continuous compression experiment was performed in the rectangular reservoir of the "zero order" trough.[19]

All the intermediates and final products gave satisfactory analytical and spectroscopic data, which are described in detail in the Experimental Section. It should be noticed that in the ^{13}C NMR spectra of the 2-oxo amide derivatives **7a**,**b** and **8a**,**b** two signals corresponding to carbon atoms of COCONH were clearly assigned. The carbon atom of the 2-oxo group was shifted at 199 ppm due to the presence of the adjacent amide function, while the signal of the amide group carbon atom appeared at 161 ppm. Furthermore, ^{13}C NMR spectra of **7a**,**b** showed a signal at 172 ppm, which was assigned to the ester group carbon atom. Two signals corresponding to the carbon atoms of 1,3-diaminopropan-2-ol backbone appeared at 70 ppm (*C*HOCO) and 39 ppm (CH_2NH).

Force/Area Curves of Bis-2-oxo Amide Triacylglycerol Analogues. The use of the monolayer technique, which is based upon surface pressure decrease owing to lipid-film hydrolysis, is advantageous for the study of lipases inhibitors since with conventional emulsified systems it is not possible to control the "interfacial quality".[17] The kinetic studies of the lipase hydrolysis reactions require that the lipids used form a stable monomolecular film at the air/water interface.[18]

To determine the film stability and the interfacial properties at the air/water interface of bis-2-oxo amide derivatives synthesized, we recorded their force/area curves. The experiments were performed in the reservoir compartment of a "zero-order" trough. A force/area curve was obtained after a small volume of lipid solution, in a volatile solvent, was spread 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.

Figure 1 shows the molecular area dependency for compounds **7a**,**b** and **8a**,**b** as a function of the surface pressure of a film spread over a buffered subphase at pH 8.0. Comparing the curves obtained for the pairs of bis-2-oxo amide esters **7a**,**b** and bis-2-oxo amide ethers **8a**,**b**, one can notice a decrease in the molecular area occupied by these compounds as the alkoxy chains at the *sn*-2 position increase.

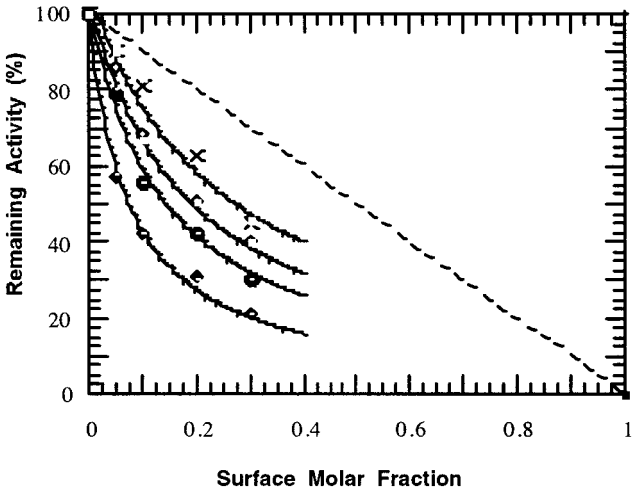

Figure 2. Effect of increasing concentrations of **7a** (◆), **7b** (◇), **8a** (●), and **8b** (×) on the remaining activity of HPL on the 1,2-dicaprin monolayer maintained at a constant surface pressure 15 mN m^{-1}. The aqueous subphase was composed of Tris/HCl 10 mM, pH 8, NaCl 150 mM, $CaCl_2$ 21 mM, EDTA 1 mM. The kinetics of hydrolysis were recorded during 15−20 min.

Pancreatic and Gastric Lipase Activity on Mixed Films Containing Bis-2-oxo Amide Triacylglycerol Analogues. The inhibition of HPL and HGL was studied by means of the monomolecular film technique[18,19] with mixed films of 1,2-dicaprin containing variable proportions of each synthetic bis-2-oxo amide triacylglycerol analogue synthesized. The inhibition studies were performed at a constant surface pressure of 15 mN m^{-1} for HPL and 20 mN m^{-1} for HGL. At 15 mN m^{-1} and 20 mN m^{-1}, HPL and HGL were, respectively, active and linear kinetics were recorded.

Remaining lipase activity was plotted as a function of the inhibitor surface molar fraction (α). Lipase hydrolysis rates of 1,2-dicaprin decreased sharply as the surface molar fraction of inhibitors increased. The data obtained for HPL and HGL are presented in Figures 2 and 3, respectively. The dotted line corresponds to surface dilution phenomena, which reflects the decrease of lipase activity that would be observed if a nonsubstrate, noninhibitor compound, that is, a so-called "surface dilutor", were present in the monomolecular film. The inhibitor surface molar fractions α_{50} obtained for all the bis-2-oxo amide triacylglycerol analogues are summarized in Table 1. The α_{50} is defined as the surface molar fraction of inhibitor which reduces by 50% the initial rate of lipolysis.

As shown from these data, the length of the *sn*-2 chain (acyloxy or alkoxy) influences the potency of the inhibition. Both HPL and HGL prefer the chain of 10 carbon atoms to the long sixteen carbon atoms chain. Specifically, a chain length decrease from 16 (**7b**, **8b**) to 10 (**7a**, **8a**) carbon atoms causes approximately a 2-fold decrease of the α_{50} values determined for both HGL and HPL. HGL seems to have a strong preference for the ester deriva-

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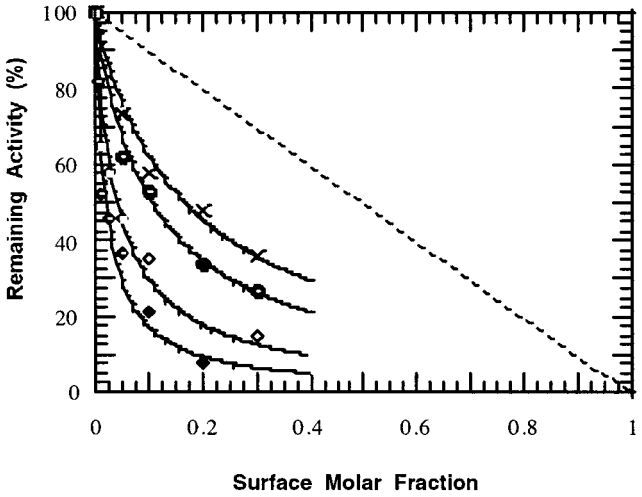

Figure 3. Effect of increasing concentrations of **7a** (◆), **7b** (◇), **8a** (●), and **8b** (×) on the remaining activity of HGL on the 1,2-dicaprin monolayer maintained at a constant surface pressure 20 mN m^{-1}. The aqueous subphase was composed of CH_3COONa 10 mM, pH 5, NaCl 150 mM, $CaCl_2$ 21 mM, EDTA 1 mM. The kinetics of hydrolysis were recorded during 15−20 min.

Table 1. Inhibition Values of Bis-2-oxo Amide Triacylglycerol Analogues on HPL and HGL with the Monolayer Technique

	α_{50}	
compd	HPL	HGL
7a	0.076 ± 0.004	0.020 ± 0.004
7b	0.173 ± 0.036	0.045 ± 0.009
8a	0.134 ± 0.015	0.109 ± 0.011
8b	0.240 ± 0.085	0.171 ± 0.019

tives of the bis-2-oxo amide triacylglycerol analogues **7a**,**b** as compared to the corresponding ether derivatives **8a**,**b**. The ester derivatives **7a**,**b** are 4- to 5-fold more potent than the corresponding inhibitors **8a**,**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,[20] while the presence of amide or carbamoyl groups at the *sn*-1 and *sn*-2 positions of inhibitors enhances the potency of *S. hyicus* lipase inhibition.[10] Among the compounds tested in this study, 2-[(2-oxohexadecanoyl)amino]-1-[[(2-oxohexadecanoyl)-amino]methyl]ethyl decanoate (**7a**) was shown to be the most potent inhibitor, causing a 50% decrease in HPL and HGL activities at 0.076 and 0.020 surface molar fractions, respectively. The α_{50} values reported for a series of chiral organophosphorus acylglycerol analogues,[21] in which one carbonyl was replaced by a phosphonate group, varied from 0.13 to 0.20 for HPL and 0.05−0.22 for HGL. Up to now, the best synthetic inhibitor of HPL reported in the literature is *O*-hexadecyl-*O*-(*p*-nitrophenyl) *n*-undecyl phosphonate, with an α_{50} value of 0.003. 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.[22] Thus, compound **7a** presents an inhibitory effect of the same order of magnitude with the most potent synthetic inhibitor of HGL. Tetrahydrolipstatin exhibits an α_{50} value of 0.0025 for HGL;[23] this registered anti-obesity drug is 10-fold more potent than 1,3-bis-2-oxo amide **7a**.

Conclusion

In conclusion, we have developed a novel class of potent human gastric lipase inhibitors. 2-Acyloxy-1,3-bis-2-oxo amide triacylglycerol analogues are easily prepared and are highly similar to real substrates from a structural point of view. The 2-oxo amide group is an efficient reactive functionality. The finding that the ester group at the *sn*-2 position contributes to the binding of the inhibitor indicates that the optimization of the structural elements of such inhibitors (for example length of 2-oxo amide moieties) could potentiate the inhibition by increasing the protein-inhibitor interactions.

Experimental Section

1,3-Diaminopropan-2-ol and AcNH−TEMPO were purchased from Aldrich. 1,2-Dicaprin was purchased from Sigma. Analytical TLC plates (silica gel 60 F_{254}) and silica gel 60 (70−230 mesh) were purchased from Merck. Visualization of spots was effected with UV light and/or phosphomolybdic acid and/or ninhydrin both in ethanol stain. HPL and HGL were purified at the laboratory using previously described procedures.[24,25] Et_2O was dried by standard procedures and stored over Na. Et_3N was distilled over ninhydrin. All other solvents and chemicals were of reagent grade and used without further purification. Melting points were determined on a Buchi 530 apparatus and are uncorrected. ^1H NMR, and ^{13}C NMR spectra were obtained in $CDCl_3$ using a Varian Mercury (200 MHz) spectrometer. Where applicable, structural assignments were based on DEPT and COSY experiments. Mass spectra were obtained on a VG Analytical ZAB-SE instrument. Elemental analyses were performed on a Perkin-Elmer 2400 instrument.

***tert*-Butyl 3-[(*tert*-Butoxycarbonyl)amino]-2-hydroxypropylcarbamate (2).** To a stirred solution of 1,3-diaminopropan-2-ol (0.09 g, 1.0 mmol) in MeOH (25 mL) were added in portions Et_3N (1.0 mL) and subsequently di-*tert*-butyl dicarbonate (0.65 g, 3.0 mmol). The reaction mixture was stirred for 20 min at 40−50 °C and for 1 h at room temperature. The solvent was evaporated under reduced pressure, and the crude product was purified by column chromatography on silica gel ($CHCl_3$/MeOH 9/1): yield 0.26 g (90%); mp 96−98 °C; ^1H NMR δ 5.35 (m, 2H), 4.05 (m, 1H), 3.75 (m, 1H), 3.20 (m, 4H), 1.45 (s, 18H); ^{13}C NMR δ 156.7, 79.2, 69.9, 43.1, 28.1. Anal. Calcd for $C_{13}H_{26}N_2O_5$: C, 53.78; H, 9.04; N, 9.65. Found: C, 53.64; H, 9.25; N, 9.46.

General Procedure for the Synthesis of 2-[(*tert*-Butoxycarbonyl)amino]-1-[*tert*-butoxycarbonyl)amino]methyl]ethyl alkanoates (3a,b). To a stirred solution of compound **2** (0.29 g, 1.0 mmol), the appropriate fatty acid (1.0 mmol), and catalytic amount of DMAP in CH_2Cl_2 (5 mL) was added dropwise DCC (0.25 g, 1.2 mmol) in CH_2Cl_2 (2 mL) at 0 °C. After being kept at 0 °C for 30 min, the reaction mixture was stirred at room temperature for 24 h, then filtered to remove the white precipitate, concentrated under reduced pressure and purified by column chromatography (petroleum ether 40−60 °C/EtOAc 7/3).

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2-[(*tert*-Butoxycarbonyl)amino]-1-[*tert*-butoxycarbonyl)amino]methyl]ethyl decanoate (3a): yield 0.32 g (73%); mp 49−51 °C; ^{1}H NMR δ 5.0 (m, 2H), 4.85 (m, 1H), 3.3 (m, 4H), 2.3 (t, 2H, J = 11.1 Hz), 1.7−1.2 (m, 32H), 0.9 (t, 3H, J = 6.5 Hz); ^{13}C NMR δ 173.1 (C), 156.1 (C), 79.5 (C), 71.5 (CH), 40.3 (CH_2), 34.2 (CH_2), 31.8 (CH_2) 29.6 (CH_2), 29.3 (CH_2), 29.2 (CH_2), 29.1 (CH_2), 28.3 (CH_3), 24.8 (CH_2), 22.6 (CH_2), 14.1 (CH_3). Anal. Calcd for $C_{23}H_{44}N_2O_6 \cdot 1.5H_2O$: C, 58.57; H, 10.04; N, 5.94. Found: C, 58.74; H, 9.95; N, 6.36.

2-[(*tert*-Butoxycarbonyl)amino]-1-[*tert*-butoxycarbonyl)amino]methyl]ethyl hexadecanoate (3b): yield 0.37 g (70%); mp 54−56 °C; ^{1}H NMR δ 5.0 (m, 2H), 4.85 (m, 1H), 3.3 (t, 4H), 2.3 (t, 2H, J = 10 Hz), 1.95−1.2 (m, 44H), 0.9 (t, 3H, J = 6.6 Hz). Anal. Calcd for $C_{29}H_{56}N_2O_6$: C, 65.87; H, 10.67; N, 5.30. Found: C, 65.52; H, 10.81; N, 5.52.

General Procedure for the Synthesis of *tert*-Butyl 2-Alkoxy-3-[(*tert*-butoxycarbonyl)amino]propylcarbamates (4a,b). To a stirred solution of compound **2** (0.29 g, 1.0 mmol) and the appropriate alkyl bromide (3.0 mmol) in benzene (5 mL), were added aqueous NaOH 50% (5 mL), and Bu_4NHSO_4 (0.085 g, 0.25 mmol) at room temperature. After vigorous stirring for 5 h at 50−60 °C, the reaction mixture was allowed to obtain the ambient temperature and EtOAc and water were added. The organic phase was washed with brine and dried (Na_2SO_4). The residue was purified by column chromatography (petroleum ether 40−60 °C/EtOAc 7/3).

***tert*-Butyl 2-decyloxy-3-[(*tert*-butoxycarbonyl)amino]-propylcarbamate (4a):** yield 0.22 g (53%); ^{1}H NMR δ 5.0 (m, 2H), 3.5 (m, 4H), 3.35 (m, 1H), 3.1 (m, 2H), 1.6−1.25 (m, 34H), 0.9 (t, 3H, J = 6.5 Hz); ^{13}C NMR δ 156.3 (C), 79.3 (C), 76.3 (CH), 69.7 (CH_2), 40.4 (CH_2), 31.8 (CH_2), 29.9 (CH_2), 29.5 (CH_2), 29.5 (CH_2), 29.4 (CH_2), 29.2 (CH_2), 28.3 (CH_3), 26.0 (CH_2), 22.6 (CH_2), 14.0 (CH_3). Anal. Calcd for $C_{23}H_{46}N_2O_5$: C, 64.15; H, 10.77; N, 6.51. Found: C, 64.04; H, 10.86; N, 6.33.

***tert*-Butyl 2-hexadecyloxy-3-[(*tert*-butoxycarbonyl)-amino]propylcarbamate (4b):** yield 0.25 g (48%); ^{1}H NMR δ 5.0 (m, 2H), 3.5−3.1 (m, 7H), 1.6−1.25 (m, 46H), 0.9 (t, 3H, J = 6.5 Hz). Anal. Calcd for $C_{29}H_{58}N_2O_5$: C, 67.66; H, 11.36; N, 5.44. Found: C, 67.48; H, 11.49; N, 5.36.

General Procedure for the Synthesis of 2-[(2-Hydroxyhexadecanoyl)amino]-1-[[(2-hydroxyhexadecanoyl)amino]methyl]ethyl Alkanoates (5a,b) and *N*-[2-Alkyloxy-3-[(2-hydroxyhexadecanoyl)amino]propyl]-2-hydroxyhexadecanamides (6a,b). Compounds **3a**,**b** and **4a**,**b** (1.0 mmol) were treated with 4 N HCl in Et_2O (16 mL) for 1 h at room temperature. The solvent and the excess acid were evaporated under reduced pressure and the residue was reevaporated twice from Et_2O. The hydrochloride salts were used directly to the next step.

To a stirred solution of 2-hydroxyhexadecanoic acid (0.54 g, 2.0 mmol) and the appropriate hydrochloride derivative of 1,3-diaminopropan-2-ol (1.0 mmol) in CH_2Cl_2 (5 mL), Et_3N (0.3 mL) and subsequently WSCI (0.57 g, 3.0 mmol) and HOBt (0.32 g, 2.0 mmol) were added at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and at room temperature for 2 days. The organic layer was washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure. The residue was purified by column chromatography ($CHCl_3$/MeOH 9:1).

2-[(2-Hydroxyhexadecanoyl)amino]-1-[[(2-hydroxyhexadecanoyl)amino]methyl]ethyl decanoate (5a): yield 0.45 g (61%); ^{1}H NMR δ 5.55 (m, 2H), 4.9 (m, 1H), 3.1−3.6 (m, 4H), 2.3 (m, 2H), 1.8−1.1 (m, 66H), 0.9 (t, 9H, J = 6.8 Hz). Anal. Calcd for $C_{45}H_{88}N_2O_6$: C, 71.76; H, 11.78; N, 3.72. Found: C, 71.65; H, 11.83; N, 3.66.

2-[(2-Hydroxyhexadecanoyl)amino]-1-[[(2-hydroxyhexadecanoyl)amino]methyl]ethyl hexadecanoate (5b): yield 0.45 g (54%); ^{1}H NMR δ 5.2 (m, 2H), 4.8 (m, 1H), 3.1−3.6 (m, 4H), 2.3 (m, 2H), 1.8−1.1 (m, 78H), 0.9 (t, 9H, J = 6.8 Hz). Anal. Calcd for $C_{51}H_{100}N_2O_6 \cdot 1H_2O$: C, 71.61; H, 12.02; N, 3.27. Found: C, 71.43; H, 12.21; N, 3.22.

***N*-[2-Decyloxy-3-[(2-hydroxyhexadecanoyl)amino]-propyl]-2-hydroxyhexadecanamide (6a):** yield 0.44 g (60%); mp 65−67 °C. Anal. Calcd for $C_{45}H_{90}N_2O_5 \cdot 1.5H_2O$: C, 70.53; H, 12.23; N, 3.65. Found: C, 70.76; H, 12.25; N, 3.32.

***N*-[2-Hexadecyloxy-3-[(2-hydroxyhexadecanoyl)amino]-propyl]-2-hydroxyhexadecanamide (6b):** yield 0.39 g (47%); mp 90−92 °C. Anal. Calcd for $C_{51}H_{102}N_2O_5 \cdot 1.5H_2O$: C, 72.03; H, 12.44; N, 3.29. Found: C, 72.13; H, 12.45; N, 2.90.

General Procedures for the Synthesis of 2-Oxo Amides (7a,b and 8a,b). Procedure A. Oxidation Using PDC. To a solution of compound **5** or **6** (1.0 mmol) in glacial acetic acid (4 mL) was added PDC (2.2 g, 6.0 mmol). After being stirred for 2 h at room temperature, the mixture was neutralized with 5% aqueous $NaHCO_3$ and extracted with EtOAc (20 mL × 3). The combined organic layers were washed with brine and dried (Na_2SO_4). The solvent was evaporated under reduced pressure, and the crude product was purified by column chromatography (petroleum ether 40−60 °C /EtOAc 7/3).

Procedure B. Oxidation Using NaOCl/AcNH−TEMPO. To a solution of compound **5** or **6** (1.0 mmol) in a mixture of EtOAc/CH_2Cl_2/toluene 1:4:3 (14 mL), were added a solution of NaBr (0.23 g, 2.2 mmol) in water (0.9 mL) and subsequently AcNH−TEMPO (4 mg, 0.02 mmol) at 0 °C. To the resulting biphasic system was added under vigorous stirring a solution of NaOCl (0.16 g, 2.2 mmol) and $NaHCO_3$ (0.17 g, 2.0 mmol) in H_2O (6 mL) dropwise at 0 °C over a period of 1 h. After the mixture was stirred for 30 min at room temperature, EtOAc (15 mL) and water (5 mL) were added. The organic layer was washed with 10% aqueous citric acid (10 mL), which contained KI (0.5 g), 10% aqueous $Na_2S_2O_3$ (10 mL), and brine and dried over Na_2SO_4. The solvent was evaporated under reduced pressure, and the crude product was purified by column chromatography (petroleum ether 40−60 °C/EtOAc 7/3).

2-[(2-Oxohexadecanoyl)amino]-1-[[(2-oxohexadecanoyl)-amino]methyl]ethyl decanoate (7a): procedure A yield 0.45 g (60%); mp 51 °C; TOF MS *m*/*z* 749 (M^+, 100), 577 (38); ^{1}H NMR δ 7.4 (m, 2H, 2 × NH), 5.0 (m, 1H, CHOCO), 3.5 (m, 4H, 2 × CH_2NH), 2.9 (t, 4H, 2 × COCOCH_2, J = 7.3 Hz), 2.3 (t, 2H, OCOCH_2, J = 6.0 Hz), 1.6−1.2 (m, 62H, 31 × CH_2), 0.9 (t, 9H, 3 × CH_3, J = 6.7 Hz); ^{13}C NMR δ 198.5 (C), 172.3 (C), 160.7 (C), 70.2 (CH), 39.4 (CH_2), 36.7 (CH_2), 34.1 (CH_2), 31.9 (CH_2), 31.8 (CH_2), 29.6 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.2 (CH_2), 29.0 (CH_2), 24.8 (CH_2), 23.1 (CH_2), 22.6 (CH_2), 14.1 (CH_3). Anal. Calcd for $C_{45}H_{84}N_2O_6 \cdot 1H_2O$: C, 70.45; H, 11.30; N, 3.65. Found: C, 70.28; H, 11.42; N, 3.86.

2-[(2-Oxohexadecanoyl)amino]-1-[[(2-oxohexadecanoyl)-amino]methyl]ethyl hexadecanoate (7b): procedure B yield 0.43 g (51%); mp 58−59 °C; TOF MS *m*/*z* 833 (M^+, 100), 577 (40); ^{1}H NMR δ 7.4 (m, 2H, 2 × NH), 5.0 (m, 1H, CHOCO), 3.45 (m, 4H, 2 × CH_2NH), 2.9 (t, 4H, 2 × COCOCH_2, J = 8.0 Hz), 2.3 (t, 2H, OCOCH_2, J = 6.6 Hz), 1.6−1.2 (m, 74H, 37 × CH_2), 0.9 (t, 9H, 3 × CH_3, J = 6.5 Hz); ^{13}C NMR δ 198.5 (C), 173.1 (C), 160.7 (C), 70.2 (CH), 39.4 (CH_2), 36.7 (CH_2), 34.1 (CH_2), 31.9 (CH_2), 29.6 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.0 (CH_2), 24.8 (CH_2), 23.1 (CH_2), 22.6 (CH_2), 14.1 (CH_3). Anal. Calcd. for $C_{51}H_{96}N_2O_6 \cdot 1.5H_2O$: C, 71.19; H, 11.60; N, 3.05. Found: C, 71.10; H, 11.65; N, 2.69.

***N*-[2-Decyloxy-3-[(2-oxohexadecanoyl)amino]propyl]-2-oxohexadecanamide (8a):** procedure A yield 0.30 g (41%); mp 61−63 °C; ^{1}H NMR δ 7.4 (m, 2H, 2 × NH), 3.8−3.4 (m, 7H, CHO, CH_2O, 2 × CH_2NH), 2.9 (t, 4H, 2 × COCOCH_2, J = 8.8 Hz), 2.0−1.2 (m, 64H, 32 × CH_2), 0.9 (t, 9H, 3 × CH_3, J = 6.8 Hz); ^{13}C NMR δ 198.6 (C), 160.6 (C), 75.7 (CH), 70.1 (CH_2), 39.6 (CH_2), 36.7 (CH_2), 31.8 (CH_2), 29.8 (CH_2), 29.6 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.0 (CH_2), 26.0 (CH_2), 23.1 (CH_2), 22.6 (CH_2), 14.1 (CH_3). Anal. Calcd for $C_{45}H_{86}N_2O_5 \cdot 0.5H_2O$: C, 72.63; H, 11.78; N, 3.42. Found: C, 72.39; H, 12.03; N, 3.07.

***N*-[2-Hexadecyloxy-3-[(2-oxohexadecanoyl)amino]-propyl]-2-oxohexadecanamide (8b):** procedure A yield 0.27 g (32%); procedure B yield 0.46 g (55%); ^{1}H NMR δ 7.4 (m, 2H, 2 × NH), 3.6−3.2 (m, 7H, 2 × CH_2NH, OCH_2, CHO), 2.9 (t, 4H, 2 × COCOCH_2, J = 8.0 Hz), 1.9−1.2 (m, 76H, 38 × CH_2), 0.9 (t, 9H, 3 × CH_3, J = 7.2 Hz); ^{13}C NMR δ 198.6 (C), 160.6 (C), 70.1 (CH), 39.5 (CH_2), 36.7 (CH_2), 31.8 (CH_2), 29.8 (CH_2), 29.6 (CH_2), 29.4 (CH_2), 29.3 (CH_2), 29.0 (CH_2), 26.0 (CH_2), 23.1 (CH_2), 22.6 (CH_2), 14.1 (CH_3). Anal. Calcd for $C_{51}H_{98}N_2O_5 \cdot 2H_2O$: C, 71.61; H, 12.02; N, 3.27. Found: C, 71.26; H, 12.11; N, 3.26.

Monomolecular Film Experiments. Force/Area Curves. Surface pressure−area curves were measured in the rectangular reservoir compartment of the "zero order" trough (14.8 cm wide and 24.9 cm long). Before each experiment the trough was at first washed with tap water, then gently brushed in the presence of distilled ethanol, washed again with plenty of tap water, and finally rinsed with double-distilled water. The lipidic film as a solution in $CHCl_3$ (approximately 1 mg mL^{-1}) was spread with a Hamilton syringe over an aqueous subphase of Tris/HCl 10 mM, pH 8.0, NaCl 100 mM, $CaCl_2$ 21 mM, EDTA 1 mM. The above buffer solution was prepared with double-distilled water and filtered through a 0.22 μm Millipore membrane. Before each utilization, residual surface-active impurities were removed by sweeping and suction of the surface.[19] The force/area curves were automatically recorded upon a continuous compression rate at 4.8 cm min^{-1}.

Enzymes Kinetics Experiments. The inhibition experiments were performed using the monolayer technique. The surface pressure of the lipid film was measured using the platinum Wilhelmy plate technique coupled with an electromicrobalance. The principle of this method has been described previously by Verger et al.[19]

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 pure films of substrate are spread. The two compartments are connected to each other by narrow surface channels. HPL (final concentration 9.9 ng mL^{-1}) and HGL (final concentration 160 ng mL^{-1}) were injected into the subphase of the reaction compartment, where efficient stirring was applied. In the case of HPL the aqueous subphase was composed of Tris/HCl 10 mM, pH 8.0, NaCl 100 mM, $CaCl_2$ 21 mM, EDTA 1 mM. In the case of HGL the aqueous subphase was composed of CH_3-COONa/HCl 10 mM, pH 5.0, NaCl 100 mM, $CaCl_2$ 21 mM, EDTA 1 mM. 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 and thus keep the surface pressure constant. The surface pressure was measured on the reservoir compartment. The surface of the reaction compartment was 100 cm^2 and its volume 120 mL. The reservoir compartment was 14.8 cm wide and 24.9 cm long. The lipidic films were spread from a chloroform solution (approximately 1 mg mL^{-1}). The kinetics were recorded for 20 min. In all cases linear kinetics were obtained. Each experiment was duplicated.

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