183. Synthesis of New Glycerolipids Linked to Hydroxamate Derivatives Designed for Two-Dimensional Crystallization of Aminopeptidase M

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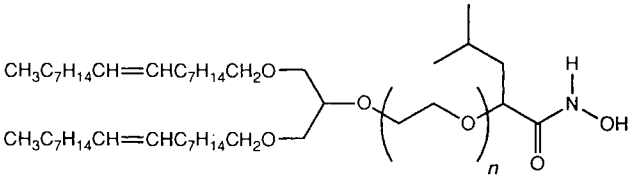
The synthesis of glycerolipids linked to hydroxamate derivatives designed for two-dimensional crystallization of aminopeptidase M is reported. The lipid moieties are readily obtained using a convergent pathway. Their structure allows the introduction of a wide variety of ligands of biological interest.

Introduction. – For a few years now, the two-dimensional (2D) crystallization technique has become a very useful technique for the structure analysis of proteins with resolutions comparable in some cases to those reached by X-ray crystallography analysis (*cf.* [1] and ref. cit. therein). The technique, using lipid bearing a specific ligand of the protein spread into monolayers, allows the study of high-molecular-weight proteins (600 kD and more) with only a few micrograms of partially purified biological material. In this way, it is particularly useful for the structural study of proteins which are available only in tiny amounts, or which are difficult to purify. Recently, we have described the synthesis of phospholipids rationally designed for 2D crystallization of progesterone and estradiol receptors [2], as well as DNA gyrase [3]. Now, using lipids not carrying phosphate groups, we attempt to employ this crystallization technique to study the mammalian aminopeptidase M (AP-M).

AP-M is a membrane-bound aminoacyl peptidase involved in peptide degradation. This protease is a 280 kD homodimer (trypsin-solubilized enzyme). Each subunit contains one Zn-atom within the active site [4]. Because of the high molecular weight of the protein, crystallization of AP-M remains a real challenge, and the 2D crystallization technique should be of value in this case. The aminopeptidase M can be reversibly inhibited by several classes of compounds, including commercially available peptides, for example bestatin or amastatin [5]. However, much simpler structures such as nonpeptidic hydroxamic acids exhibit comparable affinity towards this metalloprotease [6].

Design of the Novel Compounds. – The hydroxamic moiety is a metal-chelating group known to interact with the Zn ion within the active site. The easy preparation and manipulation of hydroxamic-acid derivatives, as compared to peptides, render these simple structures attractive candidates as specific ligands for 2D-crystallization experiments. In addition, AP-M has a broad substrate specificity, but hydrophobic amino acids



1n $n = 2$–4

such as leucine or phenylalanine residues are prefered at P_1' [4] (*Schecter* and *Berger* terminology [7]). With the aim to make a structural study of aminopeptidase M using the 2D crystallization technique, we have designed and synthesized original structures utilizing the cumulative effect of a metal-coordinating moiety and the substrate specificity at P_1'.

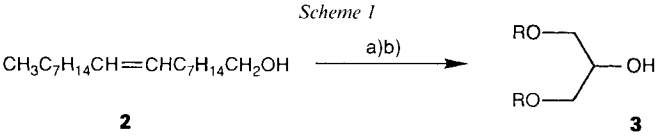
Because the hydrophilic nature of such a hydroxamic-acid ligand, the presence of a phosphate group in the lipid molecule is not necessary to ensure amphiphilic character. For the lipophilic part of the molecule, we used a glycerol dialkenyl structure.

Biophysical properties required of the lipids for 2D crystallization of proteins on lipid layers at the air/water interface are fulfilled with symmetrical 1,3-dioleyl-glycerol derivatives (these properties, such as fluidity and monolayer stability, are essentially determined by the nature of the fatty chains) [8]. To be able to adjust the accessibility of the ligand for the protein [1], we introduced two, three, or four oxyethylene units between the glycerol backbone and the specific ligand. The ligand elaborated was an α-isobutylhydroxamic-acid derivative, the hydrophobic side chain at P_1' improving the affinity for AP-M. To avoid stereochemical problems, the α-isobutylhydroxamate moiety is introduced without stereochemical control. Due to the amphiphilic behavior of compounds $\mathbf{1}_n$, we did not succeed in determining accurate affinity constants for the AP-M. However, substitution of the 1,3-dioleyl-glycerol moiety by a $PhCH_2$ group leads to a water-soluble compound which could be tested and displays competitive inhibition towards AP-M in the same range as bestatin (K_i 10^{-6} M [6]). Moreover, omission of the i-Bu chain decreases the affinity by two orders of magnitude. The specific interactions of compounds $\mathbf{1}_n$ with AP-M are of interest and should be strong enough to be used in 2D-crystallization experiments.

In this paper, we describe the synthesis of those lipids $\mathbf{1}_n$ designed for structural analysis of the AP-M.

Syntheses. – Lipids $\mathbf{1}_n$ have been synthesized using a convergent pathway. Three fragments are separately constructed: the lipid backbone **3** (*Scheme 1*), the spacer units $\mathbf{5}_n$ (*Scheme 2*), and an α-bromo-ester **7** conveniently prepared from D,L-leucine according to reference [9] (*Scheme 3*). The fragments are brought together in the final steps of the synthesis. The 1,3-dioleyl-glycerol moiety is obtained in a one-step procedure by reacting

Scheme 1



2 **3**

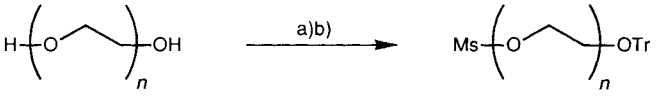
R = $CH_3C_7H_{14}$=$CHC_7H_{14}CH_2$

a) NaH, THF, HMPA. b) Epichlorohydrin.

epichlorohydrin with 2 equiv. of oleyl alcohol (**2**) in the presence of NaH affording the desired symmetrical dialkylglycerol ether **3** in 58% yield (*Scheme 1*). The regiospecificity of this one-pot synthesis is to be emphasized, since no 1,2-isomer could be detected by ^{1}H-NMR of the crude product.

Poly(oxyethylene) units are suitable for the elaboration of the various spacer lengths in order to provide this moiety of the molecule with hydrophilic properties. The spacers $\mathbf{5}_n$ are readily obtained *via* a two-steps sequence starting from di-, tri-, or tetraethylene glycol $\mathbf{4}_n$ (*Scheme 2*). Reaction of diols $\mathbf{4}_n$ with triphenylmethanol and a catalytic amount of TsOH [10] results in monoprotection and subsequent conversion of the remaining OH group into a MsO group leads to crystalline derivatives $\mathbf{5}_n$ (*Scheme 2*).

Scheme 2

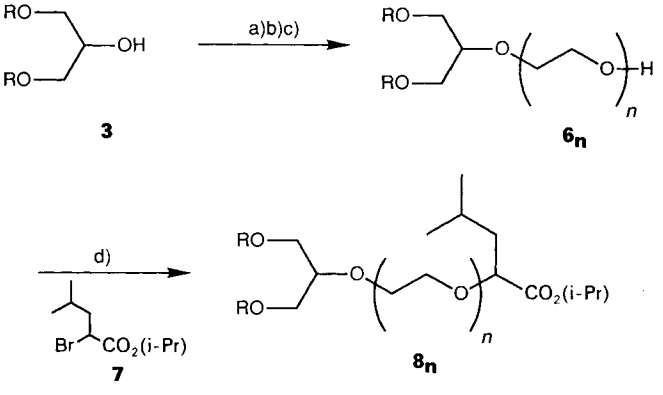


$\mathbf{4_n}$ $\mathbf{5_n}$

n = 2–4. a) TrOH, TsOH. b) MsCl, Et_3N.

The spacers are linked to the glycerol backbone by condensing $\mathbf{5}_n$ with the sodium alkoxide of **3**. The nucleophilic displacement by the hindered secondary alcohol **3** is readily achieved in refluxing THF with hexamethylphosphoric triamide (HMPA) as co-solvent. Subsequent removal of the Tr group under acidic conditions is effected on the crude product to afford the corresponding primary alcohol $\mathbf{6}_n$ in fairly good yield (*Scheme 3*). Finally, the α-isobutylacetate moiety was introduced by displacing the

Scheme 3



3 $\mathbf{6_n}$ **7** $\mathbf{8_n}$

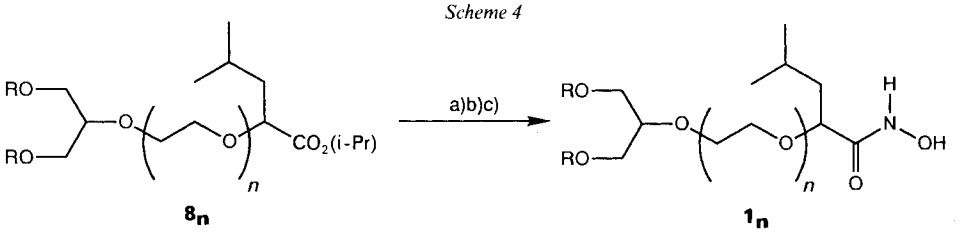
n = 2–4. a) NaH, THF, HMPA. b) **5n**. c) TsOH, MeOH. d) NaH, THF, HMPA.

bromide on isopropyl (*RS*)-2-bromo-4-methylpentanoate **7** [9] with the sodium alkoxide of $\mathbf{6}_n$ at −20° to avoid elimination and transesterification side reactions (*Scheme 3*).

The highly hydrophobic carboxylate $\mathbf{8}_n$ is converted into the corresponding hydroxamic acid $\mathbf{1}_n$ in a two-steps sequence (*Scheme 4*). Because of their lipophilic character,

saponification of compounds $\mathbf{8}_n$ was carried out in MeOH/BuOH at 50° under sonication. Commonly used methods for the synthesis of hydroxamic acids are the direct acylation of hydroxylamine with various acylating reagents, such as carboxylic acid chlorides or activated esters (*cf.* [6] and ref. cit. therein). To avoid solubility and *O*-acylation problems, we used *O*-[(*tert*-butyl)dimethylsilyl]hydroxylamine; this reagent was primarily described for preparation of oximes [11]. With oxalyl chloride, we did not succeed in activating the carboxylic acid, neither in CH_2Cl_2 nor in benzene. The coupling procedure using *N*,*N'*-dicyclohexylcarbodiimide (DDC) and 1-hydroxybenzotriazole (HOBT) proved to be efficient in this case. The resulting *O*-silylated hydroxamic acid was not isolated but was allowed to react with AcOEt and CsF [6] to give the free hydroxamic acids $\mathbf{1}_n$ in good yields (*Scheme 4*).

Scheme 4



$\mathbf{8_n}$ $\mathbf{1_n}$

n = 2–4. a) LiOH, H_2O, MeOH, n/BuOH,)))). b) DCC, HOBT, NH_2–OTBDMS. c) AcOH, CsF.

Conclusion. – Successful 2D crystallization of proteins on lipid films have mostly been performed using glycerophospholipid derivatives [1]. In the case of water-soluble ligands, the presence of the phosphate group in the lipid structure is not necessary. The ligand provides the molecule with the amphiphilic properties required for spreading of the lipids into a monolayer at the air/water interface. In practice, synthesis and manipulation of such phosphate-lacking molecules were found to be much more easy than in the case of phospholipids. In addition, it is noteworthy that the lipid structure $\mathbf{6}_n$ developed here allows the use of a wide variety of chemical reactions to introduce different ligands of biological interest. The primary OH group can react as a nucleophile under appropriate conditions or can be easily transformed into a leaving group to be displaced by a nucleophile.

Attention is now directed to biophysical studies of the developed compounds spread into monolayers at the air/water interface in order to produce 2D crystals of the AP-M.

Experimental Part

General. All reactions with air- or moisture-sensitive reactants and solvents were carried out in oven- or flame-dried glassware under a positive pressure of dry Ar. THF was distilled over Na/benzophenone and CH_2Cl_2 over CaH_2, just before use. Reactions were monitored by TLC (*Merck*, precoated plates 0.25 mm, silica gel *60*, *F 254*); solvent ratios are given in *v/v*. Products were purified by flash chromatography (FC; *Merck*, silica gel *60*, 0.040–0.063 mm, 230–400 mesh ASTM). M.p.: *Büchi 535* apparatus. IR (ν in cm^{-1}): *Bruker IFS 66*. ^1H- and ^{13}C-NMR spectra: *Bruker-WP-200-Sy* spectrometer; chemical shifts δ in ppm relative to TMS (0.00 ppm) and $CHCl_3$ (7.27 ppm), coupling constants J in Hz. Mass spectra (chemical ionization, NH_3): *Finnigan TSQ 46*.

*4-{2-{2-[(*RS*)-Bis(oleyloxy)methoxy]ethoxy}ethoxy}-*N*-hydroxy-2-methylpentanamide* (**1_2**). A mixture of **8_2** (0.42 g, 0.5 mmol) and $LiOH \cdot H_2O$ (0.08 g, 2.0 mmol) in MeOH (4.0 ml), BuOH (2.0 ml), and H_2O (1.0 ml) was sonicated 8 h in a sonicating bath heated at 50°. The mixture was brought to pH 2–3 with 10% aq. HCl soln. (5.0 ml) and extracted with AcOEt (3 × 20.0 ml). The combined org. layers were dried ($MgSO_4$) and evaporated *in vacuo*. The residue was taken up into CH_2Cl_2 (3.0 ml), and to this soln. was added HOBT (0.07 g, 0.5 mmol). To the resulting suspension was added a soln. of DDC (0.11 g, 0.55 mmol) in CH_2Cl_2 (1.0 ml) at 0°. The mixture was stirred for 0.5 h at 0°, and a soln. of *O*-[(*tert*-butyl)dimethylsilyl]hydroxylamine (0.11 g, 75 mmol) and 4-(dimethylamino)pyridine (DMAP; 0.006 g, 0.05 mmol) in CH_2Cl_2 (2.0 ml) was added dropwise at 0°. The suspension was stirred at 0° for 4 h, AcOH (0.3 ml), CsF (0.16 g, 1.0 mmol), and CH_2Cl_2 (20.0 ml) were successively added. The mixture was allowed to warm to r.t. and stirred for an additional h. The precipitate was filtered, the filtrate washed with H_2O (5.0 ml), and dried ($MgSO_4$). The solvent was evaporated and the residue purified by FC (toluene/i-PrOH 97:3) to yield **1_2** as a colorless oil (0.28 g, 70%), giving a positive test with acetone $FeCl_3$ soln. according to *Fink* and *Fink* [12]. TLC (toluene/i-PrOH 95:5): R_f 0.30. IR (liquid film): 3300.0, 2924.9, 2854.4, 1670.3, 1465.8, 1367.7, 1300.0, 1250.0, 1118.9, 966.7. ^1H-NMR ($CDCl_3$, 200 MHz): 10.35–10.25 (*m*, 1 H); 8.35–8.25 (*m*, 1 H); 5.40–5.30 (*m*, 4 H); 3.90–3.40 (*m*, 18 H); 2.15–1.10 (*m*, 59 H); 1.00–0.80 (*m*, 12 H). ^{13}C-NMR ($CDCl_3$, 50 MHz): 169.63; 129.78; 129.69; 78.96; 78.34; 71.65; 70.80; 70.71; 70.57; 70.19; 70.05; 69.31; 42.17; 32.47; 31.79; 31.67; 29.57; 29.54; 29.40; 29.19; 27.10; 25.97; 24.52; 23.20; 22.55; 21.47; 13.95. MS: 809 ($C_{49}H_{95}O_7N$), 810 ($M H^+$), 824 ($M NH_4^+$).

Compounds **1_3** and **1_4** were prepared in analogy to **1_2**, starting from **8_3** and **8_4**, respectively.

*4-{2-{2-{2-[(*RS*)-Bis(oleyloxy)methoxy]ethoxy}ethoxy}ethoxy}-*N*-hydroxy-2-methylpentanamide* (**1_3**). Yield 66%. Colorless oil giving a positive test with acetone $FeCl_3$ soln. TLC (CH_2Cl_2/MeOH 1:9): R_f 0.50. IR (liquid film): 3300.0, 2924.7, 2854.4, 1675.9, 1465.8, 1367.7, 1300.2, 1250.0, 1119.8, 966.5. ^1H-NMR ($CDCl_3$, 200 MHz): 10.40–10.30 (*m*, 1 H); 7.80–7.50 (*m*, 1 H); 5.40–5.30 (*m*, 4 H); 3.85–3.40 (*m*, 22 H); 2.20–1.10 (*m*, 59 H); 1.10–0.80 (*m*, 12 H). ^{13}C-NMR ($CDCl_3$, 50 MHz): 169.60; 129.89; 129.80; 78.50; 78.41; 71.66; 70.84; 70.59; 70.49; 70.30; 70.21; 70.04; 69.92; 42.17; 32.44; 31.89; 29.76; 29.68; 29.51; 29.29; 27.19; 24.62; 23.32; 22.65; 21.47; 14.09. MS: 853 ($C_{51}H_{99}O_8N$), 854 ($M H^+$).

*4-{2-{2-{2-{2-[(*RS*)-Bis(oleyloxy)methoxy]ethoxy}ethoxy}ethoxy}ethoxy}-N-hydroxy-2-methylpentanamide* (**1_4**). Yield 64%. Colorless oil giving a positive test with acetone $FeCl_3$ soln. TLC (CH_2Cl_2/MeOH 1:9): R_f 0.40. IR (liquid film): 3300.0, 2925.2, 2854.4, 1671.7, 1465.7, 1367.7, 1350.0, 1301.4, 1249.1, 1117.1, 966.2. ^1H-NMR ($CDCl_3$, 200 MHz): 10.40–10.30 (*m*, 1 H); 7.90–7.60 (*m*, 1 H); 5.40–5.30 (*m*, 4 H); 3.90 (*dd*, J = 9.4, 3.7, 1 H); 3.80–3.35 (*m*, 25 H); 2.20–1.00 (*m*, 59 H); 1.00–0.80 (*m*, 12 H). ^{13}C-NMR ($CDCl_3$, 50 MHz): 169.87; 129.85; 129.77; 78.98; 78.39; 71.57; 70.69; 70.58; 70.51; 70.34; 70.20; 70.09; 69.64; 42.24; 32.56; 31.67; 31.75; 29.73; 29.62; 29.49; 29.27; 28.94; 27.17; 26.09; 24.55; 23.30; 22.63; 21.50; 14.07. MS: 897 ($C_{53}H_{103}O_9N$), 898 ($M H^+$).

*[(2*RS*)-1,3-Bis(oleyloxy)propan-2-ol* (**3**). A soln. of *oleyl alcohol* (**2**) (65%; 5.90 g, 22.0 mmol) in THF (10.0 ml) was added dropwise to a suspension of NaH (60% in oil; 0.97 g, 24.0 mmol) in THF (11.0 ml) and hexamethylphosphoric triamide (HMPA) (1.0 ml) at 0°. After refluxing for 1 h, the mixture was cooled to 0°, and a soln. of epichlorohydrin (1.02 g, 11.0 mmol) in THF (5.0 ml) was added dropwise. The soln. was refluxed for 18 h, added to a sat. aq. NH_4Cl soln. (20.0 ml) and extracted with AcOEt (3 × 50.0 ml). The combined org. layers were evaporated, and the crude product was purified by FC (AcOEt/hexane 1:9) to give **3** as a colorless oil (3.30 g, 58%). TLC (Et_2O/hexane 1:1): R_f 0.70. ^1H-NMR ($CDCl_3$, 200 MHz): 5.40–5.25 (*m*, 4 H); 3.95–3.70 (*m*, 1 H); 3.55–3.35 (*m*, 8 H); 2.10–1.90 (*m*, 8 H); 1.70–1.50 (*m*, 4 H); 1.50–0.95 (*m*, 44 H); 0.95–0.75 (*m*, 6 H). ^{13}C-NMR ($CDCl_3$, 50 MHz): 129.88; 129.76; 71.70; 71.63; 69.45; 31.89; 30.13; 30.09; 30.02; 29.75; 29.68; 29.61; 29.50; 29.47; 29.42; 29.29; 28.94; 28.88; 27.18; 26.09; 22.64; 14.05. MS: 592 ($C_{39}H_{76}O_3$), 594 ($M H^+$), 610 ($M NH_4^+$).

2-[2-(Triphenylmethoxy)ethoxy]ethyl Methanesulfonate (**5_2**). To a soln. of TsOH (0.19 g, 1.0 mmol) in diethyleneglycol (**4_2**; 14.2 ml, 150.0 mmol) and benzene (100.0 ml) at r.t. was added Ph_3COH (3.90 g, 15.0 mmol). After refluxing for 2 h, the solvent was evaporated. The residue was taken up in AcOEt (100.0 ml), washed with brine (3 × 50.0 ml), dried ($MgSO_4$), and evaporated *in vacuo*. The crude product was taken up in CH_2Cl_2 (20.0 ml) and Et_3N (2.36 ml, 17.0 mmol). To this soln. was added MsCl (1.27 ml, 16.5 mmol) at 0°. After 2 h, the soln. was

allowed to warm to r.t. and stirred overnight. The precipitate was filtered, the filtrate evaporated, and the crude product was purified by FC (AcOEt/hexane 3:7) to yield **5₂** as a white solid (5.4 g, 85%) which was crystallized from AcOEt/hexane as white crystals (4.8 g, 75%). M.p. 67.5°. TLC (AcOEt/hexane 1:1): R_f 0.75. IR (KBr): 3022.0, 2933.3, 2886.9, 2823.3, 1490.2, 1450.4, 1374.2, 1358.5, 1328.6, 1172.7, 1154.9, 1136.7, 1092.9, 1086.2, 1011.6, 928.4, 764.7, 711.7, 631.0, 529.1. ^{1}H-NMR ($CDCl_3$, 200 MHz): 7.55–7.25 (*m*, 15 H); 4.45–4.35 (*m*, 2 H); 3.85–3.75 (*m*, 2 H); 3.71 (*t*, *J* = 6.8, 2 H); 3.30 (*t*, *J* = 6.2, 2 H); 3.05 (*s*, 3 H). ^{13}C-NMR ($CDCl_3$, 50 MHz): 143.92; 128.62; 127.74; 126.97; 86.66; 70.80; 69.21; 69.04; 63.25; 37.66. MS: 426 ($C_{24}H_{26}O_5S$), 427 (MH^+), 444 (MNH_4^+). Anal. calc. for $C_{24}H_{26}O_5S$ (426.53): C 67.58, H 6.14; found: C 67.75, H 6.12.

Compounds **5₃** and **5₄** were prepared in analogy to **5₂**, starting from **4₃** and **4₄**, respectively.

2-{2-[2-(Triphenylmethoxy)ethoxy]ethoxy}ethyl Methanesulfonate (**5₃**). Yield 75%. Crystallization from AcOEt/hexane. White crystals. M.p. 93.5°. TLC (AcOEt/hexane 1:1): R_f 0.55. IR (KBr): 3021.0, 2932.9, 2887.0, 2823.9, 1490.0, 1450.0, 1374.5, 1356.5, 1329.0, 1172.5, 1153.5, 1133.4, 1093.9, 1085.2, 1010.5, 922.0, 768.9, 718.8, 712.3, 529.1. ^{1}H-NMR ($CDCl_3$, 200 MHz): 7.55–7.25 (*m*, 15 H); 4.45–4.35 (*m*, 2 H); 3.85–3.75 (*m*, 2 H); 3.75–3.65 (*m*, 6 H); 3.26 (*t*, *J* = 6.2, 2 H); 3.00 (*s*, 3 H). ^{13}C-NMR ($CDCl_3$, 50 MHz): 143.82; 128.67; 127.70; 86.70; 70.80; 69.22; 69.21; 63.30; 37.70. MS: 470 ($C_{26}H_{30}O_6S$), 471 (MH^+), 488 (MNH_4^+). Anal. calc. for $C_{26}H_{30}O_6S$ (470.58): C 66.36, H 6.42; found: C 66.39, H 6.37.

2-{2-{2-[2-(Triphenylmethoxy)ethoxy]ethoxy}ethoxy}ethyl Methanesulfonate (**5₄**). Yield 87%. White solid. M.p. 38.5°. TLC (AcOEt/hexane 1:1): R_f 0.35. IR (KBr): 3086.2, 3059.9, 3022.0, 3000.0, 2871.9, 1490.3, 1449.1, 1353.0, 1175.6, 1134.7, 1109.9, 1092.0, 1032.2, 1014.2, 972.0, 920.4, 763.8, 708.2, 698.8, 528.0. ^{1}H-NMR ($CDCl_3$, 200 MHz): 7.55–7.25 (*m*, 15 H); 4.40–4.30 (*m*, 2 H); 3.80–3.65 (*m*, 12 H); 3.27 (*t*, *J* = 6.5, 2 H); 3.00 (*s*, 3 H). ^{13}C-NMR ($CDCl_3$, 50 MHz): 143.99; 128.56; 127.59; 86.41; 70.63; 70.52; 70.46; 69.08; 68.82; 63.30; 37.42. MS: 514 ($C_{28}H_{34}O_7S$), 515 (MH^+), 532 (MNH_4^+). Anal. calc. for $C_{28}H_{34}O_7S$ (514.64): C 65.35, H 6.66; found: 65.70, H 6.75.

2-{2-[(RS)-Bis(oleyloxy)methoxy]ethoxy}ethanol (**6₂**). A soln. of **3** (2.45 g, 4.1 mmol) in THF (4.0 ml) was added dropwise to a suspension of NaH (60% in oil; 0.20 g, 4.9 mmol) in THF (3.0 ml) and HMPA (1.0 ml) at 0°. After refluxing for 1 h, the mixture was cooled to 0°, and a soln. of **5₂** (1.93 g, 4.50 mmol) in THF (4.0 ml) was added dropwise. The soln. was refluxed for 4 h, then added to a sat. aq. NH_4Cl soln. (20.0 ml) and extracted with AcOEt (3 × 50.0 ml). The combined org. layers were evaporated *in vacuo*, and the residue was taken up into MeOH (20.0 ml). To the MeOH soln. was added TsOH (0.08 g, 0.41 mmol), and it was refluxed for 4 h, neutralized with Na_2CO_3 (0.69 g, 8.2 mmol), and evaporated *in vacuo*. The crude product was purified by FC (AcOEt/hexane 2:8) to give **6₂** as a colorless oil (2.30 g, 82%). TLC (AcOEt/hexane 2:8): R_f 0.30. ^{1}H-NMR ($CDCl_3$, 200 MHz): 5.40–5.30 (*m*, 4 H); 3.80–3.40 (*m*, 17 H); 3.00–2.90 (*m*, 1 H); 2.10–1.90 (*m*, 8 H); 1.60–1.00 (*m*, 48 H); 0.90–0.75 (*m*, 6 H). ^{13}C-NMR ($CDCl_3$, 50 MHz): 129.70; 129.61; 78.45; 72.42; 71.51; 70.86; 70.62; 69.65; 61.53; 31.76; 29.63; 29.49; 29.39; 29.15; 29.10; 25.98; 22.49; 13.67. MS: 680 ($C_{43}H_{84}O_5$), 698 (MNH_4^+).

Compounds **6₃** and **6₄** were prepared in analogy to **6₂**, starting from **5₃** and **5₄**, respectively.

2-{2-{2-[(RS)-Bis(oleyloxy)methoxy]ethoxy}ethoxy}ethanol (**6₃**). Yield 77%. Colorless oil. TLC (AcOEt/hexane 4:6): R_f 0.40. ^{1}H-NMR ($CDCl_3$, 200 MHz): 5.40–5.30 (*m*, 4 H); 3.80–3.40 (*m*, 21 H); 3.10–3.00 (*m*, 1 H); 2.10–1.90 (*m*, 8 H); 1.65–1.00 (*m*, 48 H); 0.90–0.75 (*m*, 6 H). ^{13}C-NMR ($CDCl_3$, 50 MHz): 129.76; 129.67; 78.40; 72.52; 71.50; 70.63; 70.77; 70.54; 70.35; 69.61; 61.62; 31.78; 29.66; 29.57; 29.34; 29.18; 28.83; 27.09; 26.03; 22.53; 13.91.MS: 724 ($C_{45}H_{88}O_6$), 742 (MNH_4^+).

2-{2-{2-{2-[(RS)-Bis(oleyloxy)methoxy]ethoxy}ethoxy}ethoxy}ethanol (**6₄**). Yield 80%. Colorless oil. TLC (AcOEt/hexane 1:1): R_f 0.20. ^{1}H-NMR ($CDCl_3$, 200 MHz): 5.40–5.30 (*m*, 4 H); 3.80–3.35 (*m*, 25 H); 2.90–2.80 (*m*, 1 H); 2.15–1.80 (*m*, 8 H); 1.70–1.00 (*m*, 44 H); 0.95–0.80 (*m*, 6 H). ^{13}C-NMR ($CDCl_3$, 50 MHz): 129.63; 129.54; 78.31; 72.43; 71.38; 70.72; 70.64; 70.45; 70.37; 70.21; 69.57; 61.45; 32.37; 31.67; 30.37; 30.24; 30.15; 29.82; 29.55; 29.48; 29.29; 29.07; 28.72; 28.57; 28.48; 28.29; 26.96; 25.93; 22.42; 13.81. MS: 768 ($C_{47}H_{92}O_7$), 786 (MNH_4^+).

Isopropyl 4-{2-{2-[(RS)-Bis(oleyloxy)methoxy]ethoxy}ethoxy}pentanoate (**8₂**). A soln. of **6₂** (0.68 g, 1.0 mmol) in THF (1.0 ml) was added dropwise to a suspension of NaH (60% in oil; 0.05 g, 1.2 mmol) in THF (1.0 ml) and HMPA (0.5 ml) at 0°. After refluxing for 1 h, the soln. was added dropwise to a soln. of **7** (0.50 g, 2.0 mmol) at −20°. The resulting suspension was stirred at −20° for 7 h, then added to a sat. aq. NH_4Cl soln. (20.0 ml). The mixture was extracted with AcOEt (3 × 30.0 ml). After drying ($MgSO_4$) and evaporation, the crude product was purified by FC (AcOEt/hexane 1:9) to give **8₂** as a colorless oil (0.58 g, 70%). TLC (AcOEt/hexane 2:8): R_f 0.50. IR (liquid film): 2925.1, 2854.4, 1746.2, 1729.7, 1465.9, 1373.6, 1272.8, 1196.5, 1108.3, 967.0. ^{1}H-NMR ($CDCl_3$, 200 MHz): 5.45–5.35 (*m*, 4 H); 5.20–5.05 (*m*, 1 H); 3.95 (*dd*, *J* = 9.3, 4.5, 1 H); 3.80–3.40 (*m*, 17 H); 2.20–1.05 (*m*, 59 H); 1.00–0.80 (*m*, 18 H). ^{13}C-NMR ($CDCl_3$, 50 MHz): 172.8; 129.87; 129.77; 78.51; 78.27; 71.60; 70.91; 70.80;

69.75; 69.67; 68.01; 41.76; 31.66; 30.23; 29.97; 29.74; 29.60; 29.47; 29.24; 29.12; 28.91; 27.18; 26.11; 24.49; 23.03; 22.61; 21.79; 21.71; 13.99. MS: 836 ($C_{52}H_{100}O_7$), 854 (MNH$_4^+$).

Compounds **8$_3$** and **8$_4$** were prepared in analogy to **8$_2$**, starting from **6$_3$** and **6$_4$**, respectively.

*Isopropyl 4-{2-{2-{2-[(*RS*)-Bis(oleyloxy)methoxy]ethoxy}ethoxy}ethoxy}pentanoate* (**8$_3$**). Yield 60%. Colorless oil. TLC (AcOEt/hexane 3:7): R_f 0.50. IR (liquid film): 2925.4, 2854.8, 1745.8, 1729.0, 1466.0, 1373.9, 1270.7, 1195.4, 1108.6, 966.4. ^{1}H-NMR ($CDCl_3$, 200 MHz): 5.45–5.35 (*m*, 4 H); 5.20–5.05 (*m*, 1 H); 3.90 (*dd*, J = 9.2, 4.6, 1 H); 3.80–3.40 (*m*, 21 H); 2.20–1.05 (*m*, 59 H); 1.00–0.80 (*m*, 18 H). ^{13}C-NMR ($CDCl_3$, 50 MHz): 172.79; 129.87; 129.72; 78.56; 78.30; 71.55; 70.51; 70.56; 70.45; 69.61; 69.40; 69.32; 69.29; 68.03; 67.93; 67.87; 41.69; 31.83; 30.23; 29.97; 29.60; 29.44; 29.12; 28.28; 27.13; 26.78; 26.06; 24.42; 23.01; 22.60; 21.76; 21.67; 21.48; 13.90. MS: 880 ($C_{54}H_{104}O_8$), 898 (MNH$_4^+$).

*Isopropyl 4-{2-{2-{2-{2-[(*RS*)-Bis(oleyloxy)methoxy]ethoxy}ethoxy}ethoxy}ethoxy}pentanoate* (**8$_4$**). Yield 66%. Colorless oil: TLC (AcOEt/hexane 3:7): R_f 0.45. IR (liquid film): 2925.3, 2854.7, 1745.8, 1729.5, 1465.8, 1373.9, 1278.1, 1195.9, 966.3. ^{1}H-NMR ($CDCl_3$, 200 MHz): 5.45–5.30 (*m*, 4 H); 5.20–5.05 (*m*, 1 H); 3.90 (*dd*, J = 9.3, 4.6, 1 H); 3.80–3.40 (*m*, 25 H); 2.20–1.10 (*m*, 59 H); 1.05–0.80 (*m*, 18 H). ^{13}C-NMR ($CDCl_3$, 50 MHz): 172.35; 129.50; 129.42; 78.20; 77.82; 71.21; 70.54; 70.31; 69.47; 69.38; 67.61; 41.43; 32.27; 31.60; 29.43; 29.38; 29.21; 29.00; 26.87; 25.84; 24.16; 22.79; 22.35; 21.49; 21.42; 13.77. MS: 925 ($C_{56}H_{108}O_9$), 943 (MNH$_4^+$).

REFERENCES

[1] L. Lebeau, E. Regnier, P. Schultz, J.C. Wang, C. Mioskowski, P. Oudet, *FEBS Lett.* **1990,** *267,* 38.

[2] L. Lebeau, C. Mioskowski, P. Oudet, *Chem. Phys. Lipids* **1988,** *46,* 57; *Helv. Chim. Acta* **1991,** *74,* 1697.

[3] L. Lebeau, S. Olland, P. Oudet, C. Mioskowski, *Chem. Phys. Lipids* **1992,** *62,* 93.

[4] M. Nakanishi, A. Moriyama, Y. Narita, M. Sasaki, *J. Biochem.* **1989,** *106,* 818. J.K. Mc Donald, A.J. Barrett, 'Mammalian Proteases', Academic Press, New York, 1980, p. 59–71.

[5] A.J. Barrett, G. Salven, 'Proteinase inhibitors', Elsevier, Amsterdam, 1986; D.H. Rich, B.J. Moon, S. Harbeson, *J. Med. Chem.* **1984,** *27,* 417.

[6] J.M. Altenburger, C. Mioskowski, H. d'Orchymont, D. Schirlin, C. Schalk, C. Tarnus, *Tetrahedron Lett.* **1992,** *33,* 5055.

[7] I. Schecter, A. Berger, *Biochem. Biophys. Res. Commun.* **1967,** *527,* 167.

[8] L. Lebeau, C. Mioskowski, P. Oudet, *Biochim. Biophys. Acta* **1988,** *939,* 417.

[9] M. Larchevêque, Y. Petit, *Bull. Chem. Soc. Fr.* **1989,** *1,* 130.

[10] H.A. Smith, R.J. Smith, *J. Am. Chem. Soc.* **1948,** *70,* 2400.

[11] S.E. Denmark, M.S. Dappen, N.L. Sear, R.T. Jacobs, *J. Am. Chem. Soc.* **1990,** *112,* 3466. S.E. Denmark, M.S. Dappen, *J. Org. Chem.* **1984,** *49,* 798.

[12] K. Fink, R.M. Fink, *Proc. Soc. Exp. Biol. Med.* **1949,** *70,* 654.