Synthesis and Properties of Novel Lipopeptides and Lipid Mimetics

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Abstract: Lipid mimetics, synthetic molecules that resemble natural lipids either structurally or functionally, have been developed as potential medicinal substances. They have been successfully applied in the development of drug and peptide delivery systems and for the development of inhibitors or lipid metabolizing enzymes. Phospholipase A2 is considered to be involved as the rate-limiting step in the production of lipid mediators of inflammatory responses and, as such, it has been a target for drug design. A series of lipid mimetics including lipopeptides, amides and alcohols of lipidic α-amino acids, have been tested by bulk and monolayer assay techniques. The findings suggested the direct interaction of the tested compounds with porcine pancreatic phospholipase A2. The inactivation of the enzyme occurred in a competitive manner. The most active compound 1 (2-amino-N-hexadecyl-L-hexanamide) showed an apparent IC_{50} of $12\,\mu M$ and inhibitory power $Z = 13$ in the monolayer assay. © 1997 European Peptide Society and John Wiley & Sons, Ltd.

INTRODUCTION

The discovery that natural mammalian lipids participate in cellular signalling and that lipids and their metabolites can modulate the activity of membrane receptors, ion channels and enzymes, has initiated fundamental studies of lipid structure, metabolism and function. Synthetic molecules resembling lipids either structurally or in function could be of importance for biochemical and mechanistic stu-

dies. The term 'lipid mimetics' has been adopted for analogues of natural lipids and their metabolites, that have been developed as medicinal substances [1, 2]. Lipidic amino acids and peptides have been successfully applied as mimetics in the development of drug and peptide delivery systems [3] and an adjuvant/carrier system [4]. They have also been used as starting materials for the synthesis of sphingosine and ceramide analogues [5]. This study presents the utilization of lipid mimetics in the development of novel phospholipase A2 inhibitors.

The definitive contribution of phospholipases A2 (PLA2) (EC 3.1.1.4) to the formation of natural lipid mediators of inflammation, identified the enzyme as a target for the design and synthesis of anti-inflammatory inhibitors [6, 7]. Phospholipase A2 catalyses the hydrolysis of membrane glycero-phospholipids at the *sn*-2 position of the glycerol moiety and results into the liberation of polyunsaturated fatty acids (PUFAs) such as arachidonic acid and lysoglycerolipids. Although these molecules act as

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second messengers in their own right, PUFAs are further metabolized to prostaglandins, thromboxanes and leukotrienes, and some lysophospholipids to the platelet-activating factor (PAF). All of these have been proposed as mediators of inflammatory responses [8, 9]. The search for specific, potent and bioavailable inhibitors of PLA2 has included a wide spectrum of structurally and functionally different compounds [10–12]. Phospholipid analogues designed as substrate and transition state analogue inhibitors [12, 13], natural products [14, 15] and miscellaneous compounds derived from molecular modelling and enzyme–inhibitor interaction studies [16, 17], including series of lipophilic amines [16, 18] and amides of fatty acids [19], have given significant *in vitro* and *in vivo* anti-inflammatory results in various models, but none has emerged with a potential clinical use [6, 11].

In the present study a series of novel lipidic mimetics was synthesized, characterized and tested as potential inhibitors of porcine pancreatic phospholipase A2. The series consisted of (1) amides (compounds **1–19**) and esters (compounds **20** and **21**) of α-amino acids with saturated and unsaturated linear side chains, (2) esters of α-amino alcohols (compounds **22** and **23**) and (3) derivatives of lipidic dipeptides (compounds **24–27**).

MATERIALS AND METHODS

Melting points were determined on a Buchi micro melting point apparatus and were not corrected. Specific rotations were determined with a Perkin Elmer 141 polarimeter using a 10 cm cell. ^{1}H-NMR spectra were recorded at a 500 MHz on a Bruker-AM500 spectrometer. FAB mass spectra were recorded using a VG analytical ZAB-SE instrument. L-2-Amino-hexanoic acid was purchased from Fluka, while 2-*tert*-butyloxycarbonylamino-D,L-hexadecanoic acid was prepared according to the literature [5]. All other chemicals used were purchased from Fluka and were of reagent quality. Pre-coated TLC aluminium plates (silica gel 60 F$_{254}$) were obtained from Merck.

Synthesis of Lipopeptides and Lipidic Mimetrics

The amides **4–19** were synthesized by the direct reaction of Boc-protected 2-amino-L-hexanoic acid and 2-amino-DL-hexadecanoic acid with the corresponding lipidic amines by the water-soluble carbodiimide/HOBt method. The Boc group was removed

from all the derivatives by treatment with 4N HCl/THF. The known compound **1** [19] was prepared for comparison purposes. Compounds **2, 3** and **20–27** were synthesized as previously described [2].

Synthesis of compounds 4, 6, 8, 10, 12, 16, 18. To a stirred solution of Boc-protected amino acid (0.41 mmol) and lipidic amine (0.41 mmol) in dichloromethane (7 ml), triethylamine (0.06 ml, 0.41 mmol), water-soluble carbodiimide (55 mg, 0.41 mmol) and 1-hydroxybenzotriazole (55 mg, 0.41 mmol) were added at 0°C. The reaction mixture was left for 1 h at 0°C and overnight at 25°C. The organic layer was washed with water (2 × 15 ml), dried (MgSO$_4$) and evaporated. Pure products were obtained after flash chromatography on silica gel using hexane–AcOEt (8:2, v/v) as eluent. Yield 65–80%.

2-(tert-Butoxycarbonylamino)-N-(cis-9-octadecenoyl) hexanamide (4). ^{1}H-NMR (500 MHz, CDCl$_3$): δ 0.88 (6H, m, 2 × CH$_3$), 1.25–1.33 (26H, m, 13 × CH$_2$), 1.41 (9H, s, (CH$_3$)$_3$C), 1.43 (2H, m, CH_2CH$_2$NHCO), 1.47 (1H, m, CHCHH(CH$_2$)$_2$CH$_3$), 1.81 (1H, m, CHCHH(CH$_2$)$_2$CH$_3$), 1.99 (4H, m, CH_2CH=CHCH_2), 3.22 (2H, m, CH_2NHCO), 3.97 (1H, m, α-CH), 4.95 (1H, b, OCONH), 5.33 (2H, m, CH=CH), 6.06 (1H, b, NHCO). MS (FAB) 482 (M + 2H, 7%), 408 (25%), 255 (100%). Anal. calcd for C$_{29}$H$_{56}$N$_2$O$_3$ (480.78): C, 72.44; H, 11.75; N, 5.83. Found C, 72.25; H, 11.56; N, 6.00.

2-(tert-Butoxycarbonylamino)-N-(cis-9-octadecenoyl) hexadecanamide (6). ^{1}H-NMR (500 MHz, CDCl$_3$): δ 0.86 (6H, t, J = 7 Hz, 2 × CH$_3$), 1.10–1.25 (46H, m, 23 × CH$_2$), 1.42 (9H, s, (CH$_3$)$_3$C), 1.48 (2H, m, CH_2CH$_2$NHCO), 1.55 (1H, m, CHCHH(CH$_2$)$_{12}$CH$_3$), 1.77 (1H, m, CHCHH(CH$_2$)$_{12}$CH$_3$), 1.98 (4H, m, CH_2CH=CHCH_2), 3.23 (2H, m, CH_2NHCO), 3.99 (1H, m, α-CH), 5.15 (1H, b, OCONH), 5.33 (2H, m, CH=CH), 6.25 (1H, b, NHCO). MS (FAB) 643 (M + Na, 5%), 521 (35%), 226 (100%). Anal. calcd for C$_{39}$H$_{76}$N$_2$O$_3$ (621.04): C, 75.43; H, 12.33; N, 4.51. Found C, 75.61; H, 12.04; N, 4.59.

2-(tert-Butoxycarbonylamino)-N-(all cis-9,12-octadecadienoyl) hexanamide (8). ^{1}H-NMR (500 MHz, CDCl$_3$): δ 0.87 (6H, m, 2 × CH$_3$), 1.25–1.36 (20H, m, 10 × CH$_2$), 1.42 (9H, s, (CH$_3$)$_3$C), 1.48 (2H, m, CH_2CH$_2$NHCO), 1.57 (1H, m, CONHCHCHH-(CH$_2$)$_2$CH$_3$), 1.84 (1H, m, CONHCHCHH(CH$_2$)$_2$CH$_3$), 2.04 (4H, m, 2 × CH_2CH=CHCH$_2$), 2.76 (2H, m,

CH=CHCH_2CH=CH), 3.23 (2H, m, CH_2NHCO), 3.99 (1H, m, α-CH), 4.97 (1H, b, OCONH), 5.35 (4H, m, 2 × CH=CH), 6.01 (1H, b, NHCO). MS (FAB) 501 (M + Na, 100%), 479 (M + H, 10%), 423 (42%), 379 (100%), 226 (35%). Anal. calcd for $C_{29}H_{54}N_2O_3$ (478.76): C, 72.75; H, 11.37; N, 5.85. Found C, 72.88; H, 11.24; N, 5.90.

2-(tert-Butoxycarbonylamino)-N-(all cis-9,12-octadecadienoyl) hexadecanamide (10). ^{1}H-NMR (500 MHz, CDCl$_3$): δ 0.86 (3H, t, $J = 7$ Hz, CH$_3$), 0.88 (3H, t, $J = 7$ Hz, CH$_3$), 1.23–1.34 (40H, m, 20 × CH$_2$), 1.43 (9H, s, (CH$_3$)$_3$C), 1.47 (2H, m, CH_2CH$_2$NHCO), 1.57 (1H, m, CHCHH(CH$_2$)$_{12}$CH$_3$), 1.78 (1H, m, CHCHH(CH$_2$)$_{12}$CH$_3$), 2.03 (4H, m, 2 × CH_2CH=CHCH$_2$CH=CH), 2.76 (2H, m, CH=CHCH_2CH=CH), 3.22 (2H, m, CH_2NHCO), 3.99 (1H, m, α-CH), 5.03 (1H, b, OCONH), 5.34 (4H, m, 2 × CH=CH), 6.21 (1H, b, NHCO). MS (FAB) 641 (M + Na, 75%), 619 (M + H, 35%), 519 (45%), 441 (100%). Anal. calcd for $C_{39}H_{74}N_2O_3$ (619.03): C, 75.67; H, 12.05; N, 4.53. Found C, 75.56; H, 11.89; N, 4.61.

2-(tert-Butoxycarbonylamino)-N-(all cis-9,12,15-octadecatrienoyl) hexanamide (12). ^{1}H-NMR (500 MHz, CDCl$_3$): δ 0.89 (3H, t, $J = 7$ Hz, CH$_3$), 0.97 (3H, t, $J = 7.5$ Hz, CH$_3$), 1.25–1.35 (14H, m, 7 × CH$_2$), 1.43 (9H, s, (CH$_3$)$_3$C), 1.58 (1H, m, CONHCHCHH(CH$_2$)$_2$CH$_3$), 1.83 (1H, m, CONHCHCHH(CH$_2$)$_2$CH$_3$), 2.05 (4H, m, 2 × CH_2CH=CHCH$_2$CH=CH), 2.80 (4H, m, 2 × CH=CHCH_2CH=CH), 3.23 (2H, m, CH_2NHCO), 3.98 (1H, m, α-CH), 4.97 (1H, b, OCONH), 5.36 (6H, m, 3 × CH=CH), 6.03 (1H, b, NHCO). MS (FAB) 477 (M + H, 15%), 421 (40%), 377 (100%). Anal. calcd for $C_{29}H_{52}N_2O_3$ (476.74): C, 73.06; H, 10.99; N, 5.88. Found C, 73.29; H, 10.74; N, 5.99.

2-(tert-Butoxycarbonylamino)-N-(all cis-9,12,15-octadecatrienoyl) hexanadecanamide (14). ^{1}H-NMR (500 MHz, CDCl$_3$): δ 0.87 (3H, t, $J = 7$ Hz, CH$_3$), 0.96 (3H, t, $J = 7.5$ Hz, CH$_3$), 1.24–1.34 (34H, m, 17 × CH$_2$), 1.43 (9H, s, (CH$_3$)$_3$C), 1.47 (2H, m, CH_2CH$_2$NHCO), 1.57 (1H, m, CHCHH(CH$_2$)$_{12}$CH$_3$), 1.80 (1H, m, CHCHH(CH$_2$)$_{12}$CH$_3$), 2.05 (4H, m, 2 × CH_2CH=CHCH$_2$CH=CH), 2.80 (4H, m, 2 × CH=CHCH_2CH=CH), 3.22 (2H, m, CH_2NHCO), 3.98 (1H, m, α-CH), 5.00 (1H, b, OCONH), 5.35 (6H, m, 3 × CH=CH), 6.08 (1H, b, NHCO). MS (FAB) 618 (M + 2H, 5%), 562 (35%), 518 (70%), 226 (100%). Anal. calcd for $C_{39}H_{72}N_2O_3$ (617.01): C 75.92; H, 11.76; N, 4.54. Found C, 76.01; H, 11.75; N, 4.39.

2-(tert-Butoxycarbonylamino)-N-(all cis-5,8,11,14-eicosanetetraenoyl) hexanamide (16). ^{1}H-NMR (500 MHz, CDCl$_3$): δ 0.88 (6H, m, 2 × CH$_3$), 1.25–1.38 (12H, m, 6 × CH$_2$), 1.44 (9H, s, (CH$_3$)$_3$C, 1.54 (3H, m, CH_2CH$_2$NHCO, CONHCHCHH(CH$_2$)$_2$CH$_3$), 1.83 (1H, m, CONHCHCHH(CH$_2$)$_2$CH$_3$), 2.06 (4H, m, 2 × CH_2CH=CHCH$_2$CH=CH), 2.82 (6H, m, 3 × CH=CHCH_2CH=CH), 3.25 (2H, m, CH_2NHCO), 3.99 (1H, m, α-CH), 4.95 (1H, b, OCONH), 5.35 (8H, m, CH=CH), 6.01 (1H, b, NHCO). MS (FAB) 503 (M + H, 10%), 403 (60%), 147 (100%). Anal. calcd for $C_{31}H_{54}N_2O_3$ (502.78): C, 74.06; H, 10.83; N, 5.57. Found C, 73.82; H, 10.64; N, 5.69.

2-(tert-Butoxycarbonylamino)-N-(all cis-5, 8, 11, 14-eicosanetetraenoyl) hexadecanamide (18). ^{1}H-NMR (500 MHz, CDCl$_3$): δ 0.87 (3H, t, $J = 7$ Hz, CH$_3$), 0.89 (3H, t, $J = 7$ Hz, CH$_3$), 1.24–1.29 (30H, m, 15 × CH$_2$), 1.39 (2H, m, CH_2CH$_2$CH$_2$NHCO), 1.43 (9H, s, (CH$_3$)$_3$C), 1.52 (3H, m, CH_2CH$_2$NHCO, CHCHH(CH$_2$)$_{12}$CH$_3$), 1.82 (1H, m, CHCHH(CH$_2$)$_{12}$CH$_3$), 2.06 (4H, m, 2 × CH_2CH=CHCH$_2$CH=CH), 2.80 (6H, m, 3 × CH=CHCH_2CH=CH), 3.24 (2H, m, CH_2NHCO), 3.98 (1H, m, α-CH), 4.95 (1H, b, OCONH), 5.36 (8H, m, CH=CH), 6.00 (1H, b, NHCO). MS (FAB) 666 (M + Na, 15%), 544 (25%), 226 (100%). Anal. calcd for $C_{41}H_{74}N_2O_3$ (643.05): C, 76.58; H, 11.60; N, 4.36. Found C, 76.31; H, 11.44; N, 4.42.

Synthesis of the Compounds 5, 7, 9, 11, 13, 15, 17, 19. Boc-protected compound (0.1 mmol) was treated with 4N HCl in THF (5 ml) at room temperature for 30 min. The excess acid and solvents were removed under reduced pressure and the residue was evaporated twice from anhydrous THF. The oily solid was triturated with cold anhydrous acetone. Yield 85–90%.

2-Amino-N-(cis-9-octadecenoyl) hexanamide hydrochloride (5). ^{1}H-NMR (500 MHz, CD$_3$OD): δ 0.89 (3H, m, CH$_3$), 0.94 (3H, t, $J = 7$ Hz, CH$_3$), 1.22–1.41 (26H, m, 13 × CH$_2$), 1.52 (2H, m, CH_2CH$_2$NHCO), 1.80 (2H, m, CH$_3$(CH$_2$)$_2$CH_2CHNH$_3^+$), 2.01 (4H, m, CH_2CH=CHCH_2), 3.15 and 3.55 (2H, m, m, CH_2NHCO), 3.73 (1H, m, CHNH$_3^+$), 5.33 (2H, m, CH=CH). MS (FAB) 381 (M + H – HCl, 40%, 86 (100%). Anal. calcd for $C_{24}H_{49}ClN_2O \cdot H_2O$ (435.13): C, 66.25; H, 11.81; N, 6.44. Found C, 66.20; H, 12.00; N, 6.24.

2-Amino-N-(cis-9-octadecenoyl) hexadecanamide hydrochloride (7). ^{1}H-NMR (500 MHz, CDCl$_3$): δ 0.87

(6H, t, $J = 7$ Hz, $2 \times CH_3$), 1.25 (46H, m, $23 \times CH_2$), 1.71 (2H, m, CH_2CH$_2$NHCO), 1.86 (2H, m, CH$_3$(CH$_2$)$_{12}$CH_2CHNH$_3^+$), 2.08 (4H, m, CH_2CH=CHCH$_2$), 3.47 (1H, m, CHNH$_3^+$), 3.68 and 3.56 (2H, m, m, CH_2NHCO), 5.33 (2H, m, CH=CH), 8.13 (1H, b, NHCO). MS (FAB) 521 (M + H − HCl, 100%), 226 (50%). Anal. calcd for $C_{34}H_{69}C1N_2O \cdot H_2O$ (575.40): C, 70.97; H, 12.44; N, 4.87. Found C, 70.64; H, 12.64; N, 4.79.

2-Amino-N-(all cis-9,12-octadecadienoyl) hexanamide hydrochloride (9). ^{1}H-NMR (500 MHz, CDCl$_3$): δ 0.87 (6H, m, $2 \times CH_3$), 1.18–1.23 (20H, m, $10 \times CH_2$), 1.71 (2H, m, CH_2CH$_2$NHCO), 1.86 (2H, m, CH$_3$(CH$_2$)$_2$CH_2CHNH$_3^+$), 2.03 (4H, m, $2 \times$ CH_2CH=CHCH$_2$), 2.75 (2H, m, CH=CHCH_2CH=CH), 3.47 (1H, m, CHNH$_3^+$), 3.56 and 3.68 (2H, m, m, CH_2NHCO), 5.32 (4H, m, CH=CH), 8.01 (1H, b, NHCO). MAS (FAB) 379 (M + H − HCl, 100%), 266 (100%). Anal. calcd for $C_{24}H_{47}C1N_2O$ (415.10): C, 69.44; H, 11.41; N, 6.75. Found C, 69.69; H, 11.67; N, 6.62.

2-Amino-N-(all cis-9,12-octadecadienoyl) hexadecanamide hydrochloride (11). ^{1}H-NMR (500 MHz, CDCl$_3$): δ 0.86 (6H, m, $2 \times CH_3$), 1.18–1.33 (40H, m, $20 \times CH_2$), 1.72 (2H, m, CH_2CH$_2$NHCO), 1.87 (2H, m, CH$_3$(CH$_2$)$_{12}$CH_2CHNH$_3^+$), 2.03 (4H, m, $2 \times$ CH_2CH=CHCH$_2$), 2.76 (2H, m, CH=CHCH_2CH=CH), 3.48 (1H, m, CHNH$_3^+$), 3.56 and 3.66 (2H, m, m, CH_2NHCO), 5.32 (4H, m, CH=CH), 8.14 (1H, b, NHCO). MAS (FAB) 519 (M + H − HCl, 100%), 266 (25%), 226 (100%). Anal. calcd for $C_{34}H_{67}C1N_2O$ (555.37): C, 73.53; H, 12.16; N, 5.04. Found C, 73.28; H, 12.31; N, 4.92.

2-Amino-N-(all cis-9,12,15-octadecatrienoyl) hexanamide hydrochloride (13). ^{1}H–NMR (500 MHz, CDCl$_3$): δ 0.88 (3H, t, $J = 7$ Hz, CH$_3$), 0.95 (3H, t, $J = 7.5$ Hz, CH$_3$), 1.24–1.33 (14H, m, $7 \times CH_2$), 1.70 (2H, m, CH_2CH$_2$NHCO), 1.87 (2H, m, CH$_3$(CH$_2$)$_2$CH_2CHNH$_3^+$), 2.04 (4H, m, $2 \times$ CH_2CH=CHCH$_2$CH=CH), 2.78 (4H, m, $2 \times$ CH=CHCH_2CH=CH), 3.48 (1H, m, CHNH$_3^+$), 3.57 and 3.67 (2H, m, CH_2NHCO), 5.34 (6H, m, $3 \times$ CH=CH), 8.33 (1H, b, NHCO). MS (FAB) 377 (M + H − HCl, 100%), 264 (25%), 219 (14%). Anal. calcd for $C_{24}H_{45}C1N_2O$ (413.09): C, 69.78; H, 10.98; N, 6.78. Found C, 69.57; H, 11.19; N, 6.69.

2-Amino-N-(all cis-9,12,15-octadecatrienoyl) hexadecanamide hydrochloride (15). ^{1}H-NMR (500 MHz, CDCl$_3$): δ 0.84 (3H, t, $J = 7$ Hz, CH$_3$), 0.93 (3H, t,

$J = 7.5$ Hz, CH$_3$), 1.15–1.25 (34H, m, $17 \times CH_2$), 1.68 (2H, m, CH_2CH$_2$NHCO), 1.83 (2H, m, CH$_3$(CH$_2$)$_{12}$CH_2CHNH$_3^+$), 2.02 (4H, m, $2 \times$ CH_2CH=CHCH$_2$CH=CH), 2.76 (4H, m, $2 \times$ CH=CHCH_2CH=CH), 3.44 (1H, m, CHNH$_3^+$), 3.64 and 3.54 (2H, m, m, CH_2NHCO), 5.31 (6H, m, CH=CH), 8.12 (1H, b, NHCO). MS (FAB) 517 (M + H − HCl, 60%), 264 (15%), 226 (100%). Anal. calcd for $C_{34}H_{65}C1N_2O$ (553.36): C, 73.80; H, 11.84; N, 5.06. Found C, 73.61; H, 12.04; N, 4.98.

2-Amino-N-(all cis-5,8,11,14-eicosanetetraenoyl) hexanamide hydrochloride (17). ^{1}H-NMR (500 MHz, CDCl$_3$): δ 0.87 (6H, m, $2 \times CH_3$), 1.24–1.35 (12H, m, $6 \times CH_2$), 1.72 (2H, m, CH_2CH$_2$NHCO), 1.87 (2H, m, CH$_3$(CH$_2$)$_2$CH_2CHNH$_3^+$), 2.04 (4H, m, $2 \times$ CH_2CH=CHCH$_2$CH=CH), 2.80 6H, m, $3 \times$ CH=CHCH_2CH=CH), 3.46 (1H, m, CHNH$_3^+$), 3.56 and 3.69 (2H, m, m, CH_2NHCO), 5.35 (8H, m, CH=CH), 8.23 (1H, b, NHCO). MS (FAB) 403 (M + H − HCl, 100%), 379 (50%), 266 (8%), 147 (85%). Anal. calcd for $C_{26}H_{47}C1N_2O \cdot H_2O$ (457.14): C, 68.31; H, 10.80; N, 6.13. Found C, 68.02; H, 11.07; N, 6.01.

2-Amino-N-(all cis-5,8,11,14-eicosanetetraenoyl) hexadecanamide hydrochloride (19). ^{1}H-NMR (500 MHz, CDCl$_3$): δ 0.85 (6H, m, $2 \times CH_3$), 1.23–1.36 (32H, m, $16 \times CH_2$), 1.68 (2H, m, CH_2CH$_2$NHCO), 1.84 (2H, m, CH$_3$(CH$_2$)$_{12}$-CH_2CHNH$_3^+$), 2.04 (4H, m, $2 \times$ CH_2CH=CHCH$_2$CH=CH), 2.79 (6H, m, $3 \times$ CH=CHCH_2CH=CH), 3.44 (1H, m, CHNH$_3^+$), 3.56 and 3.66 (2H, m, CH_2NHCO), 5.34 (8H, m, CH=CH), 8.24 (1H, b, NHCO). MS (FAB) 543 (M + H − HCl, 25%), 266 (5%), 226 (100%). Anal. calcd for $C_{36}H_{67}C1N_2O \cdot H_2O$ (597.41): C, 72.38; H, 11.64; N, 4.69. Found C, 72.04; H, 11.91; N, 4.52.

Biochemical Assays

Bulk PLA2 Assay. The inhibitory activity of the synthesized compounds was tested against porcine pancreatic phospholipase A2 (Sigma). A bulk radiometric assay was used with 6 nmol 1-stearoyl 2-(1–^{14}C) arachidonyl phosphatidyl choline (Amersham) (specific activity 10,000 dmp/mmol) as substrate. 20 µl phosphatidyl choline (solution in chloroform) was dried under nitrogen and resuspended in 10 µl dimethysulfoxide (DMSO) and 10 mM CaCl$_2$ in 25 mM Tris–HCl, pH 8. The compounds to be tested were used as DMSO solutions, and the total amount of DMSO used did not exceed

the 5% (v/v) of the assay volume (500 μl). The enzyme reaction was initiated by the addition of PLA2 in the preincubated (5 min) mixture of substrate and inhibitor (method C [21]). Incubations were performed at 37°C for 10 min. The enzyme reaction was stopped with 2 ml of the organic layer of a CHCl$_3$:MeOH:2N HCl (6:3:1, v/v) mixture. The organic layer was washed with 1 M KCl and applied to a silica gel TLC plate which was developed in a solvent system of CHCl$_3$:EtOH:Et$_3$N:H$_2$O (40:50:40:1, v/v). The radioactive reaction products were quantified on a Bertold TLC scanner and the results were expressed as a percentage of hydrolysis. Control experiments were performed to ensure that

the amount of DMSO used did not affect the enzyme activity. Initial experiments confirmed the linearity of the reaction. In all cases the hydrolysis was kept below 20%. Apparent IC$_{50}$ values, in μM, were determined graphically from plots of percentage inhibition versus log concentration of the tested compound. Each compound was tested in five different concentrations, each assay performed in duplicate. The results are summarized in Table 1.

Monolayer PLA2 Assay. Porcine pancreatic PLA2 inhibition assays using the monomolecular film technique were described previously [22]. The substrate used was 1,2-dilauroyl-*sn*-glycerol-3-phos-

Table 1 Structural Components of Lipid Mimetics and their Effect on the Activity of Porcine Pancreatic Phospholipase A2

Compound	R	R$_1$	X	Y	R$_2$	App IC$_{50}$[a] (μM)	% stimulation at 100 μM[a]	Z[b]
1	C$_4$H$_9$	H,HCl	=O	NH	C$_{16}$H$_{33}$	12.1 ± 1.3		13.3 ± 0.7
2	C$_{14}$H$_{29}$	Boc	=O	NH	C$_{14}$H$_{29}$	>100		2.0 ± 0.2
3	C$_{14}$H$_{29}$	H, HCl	=O	NH	C$_{14}$H$_{29}$		56	−0.36
4	C$_4$H$_9$	Boc	=O	NH	C$_{18}$H$_{35}$,△$_9$,*cis*			
5	C$_4$H$_9$	H, HCl	=O	NH	C$_{18}$H$_{35}$,△$_9$,*cis*	24.8 ± 7.1		2.0 ± 0.1
6	C$_{14}$H$_{29}$	Boc	=O	NH	C$_{18}$H$_{35}$,△$_9$,*cis*			
7	C$_{14}$H$_{29}$	H, HCl	=O	NH	C$_{18}$H$_{35}$,△$_9$,*cis*		15	−0.2
8	C$_4$H$_9$	Boc	=O	NH	C$_{18}$H$_{33}$,△$_{9,12}$,*cis*			
9	C$_4$H$_9$	H, HCl	=O	NH	C$_{18}$H$_{33}$,△$_{9,12}$,*cis*	>100		0.6
10	C$_{14}$H$_{29}$	Boc	=O	NH	C$_{18}$H$_{33}$,△$_{9,12}$,*cis*			
11	C$_{14}$H$_{29}$	H, HCl	=O	NH	C$_{18}$H$_{33}$,△$_{9,12}$,*cis*	>100		0.1
12	C$_4$H$_9$	Boc	=O	NH	C$_{18}$H$_{31}$,△$_{9,12,15}$,*cis*			
13	C$_4$H$_9$	H, HCl	=O	NH	C$_{18}$H$_{31}$,△$_{9,12,15}$,*cis*	10.8 ± 1.7		2.9 ± 1.6
14	C$_{14}$H$_{29}$	Boc	=O	NH	C$_{18}$H$_{31}$,△$_{9,12,15}$,*cis*			
15	C$_{14}$H$_{29}$	H, HCl	=O	NH	C$_{18}$H$_{31}$,△$_{9,12,15}$,*cis*		20	0.2
16	C$_4$H$_9$	Boc	=O	NH	C$_{20}$H$_{33}$,△$_{5,8,11,14}$,*cis*			
17	C$_4$H$_9$	H, HCl	=O	Nh	C$_{20}$H$_{33}$,△$_{5,8,11,14}$,*cis*	>100		0.01
18	C$_{14}$H$_{29}$	Boc	=O	NH	C$_{20}$H$_{33}$,△$_{5,8,11,14}$,*cis*			
19	C$_{14}$H$_{29}$	H, HCl	=O	NH	C$_{20}$H$_{33}$,△$_{5,8,11,14}$,*cis*	>100		−0.4
20	C$_4$H$_9$	H, HCl	=O	O	C$_{16}$H$_{33}$		55	−0.8
21	C$_4$H$_9$	H, HCl	=O	O	C$_{18}$H$_{35}$,△$_9$,*cis*		53	−0.3
22	C$_4$H$_9$	H, HCl	H, H	O−C=O	C$_{15}$H$_{31}$	>100		−0.8
23	C$_4$H$_9$	H, HCl	H, H	O−C=O	C$_{17}$H$_{33}$,△$_8$,*cis*		64	0.4
24	C$_4$H$_9$	Boc	=O	NH	(C$_{14}$H$_{29}$)−CH−CO$_2$Me		13	−0.9
25	C$_4$H$_9$	H, HCl	=O	NH	(C$_{14}$H$_{29}$)−CH−CO$_2$Me	20.3 ± 7.0		0.18
26	C$_4$H$_9$	H, HCl	=O	NH	(C$_{14}$H$_{29}$)−CH−CO$_2$H		190	−0.8
27	C$_4$H$_9$	H, HCl	=O	NH	(C$_{14}$H$_{29}$)−CH−CH$_2$OH	12 ± 4.9		0.4

[a] Data from the bulk radiometric assay.
[b] Data from the monolayer assay.

Table 2 Molecular Areas of the Tested Compounds at a Surface Pressure of 10 mN/m, as Calculated by the Corresponding Compression Isotherm Curves

Compound	Molecular area (Å^2/molecule) at 10 mN/m
1	54.9
2	66.7
3	51.0
5	65.4
7	54.3
9	49.1
11	43.2
13	44.5
15	40.8
17	54.6
19	48.7
20	60.8
21	45.5
22	73.2
23	35.2
24	86.4
25	92.2
26	62.4
27	74.0

phocholine (Fluka). All the tested compounds formed stable monomolecular films at the air/water interface and their 'compression isotherm curves' (surface pressure versus area per molecule) were recorded. The molecular air of every compound was calculated at a surface pressure of 10 mN/m (Table 2). The monolayer assays were performed according to the procedure described by Rasnac *et al.* [22, 23] using mixed substrate/inhibitor monomolecular films spread over the reaction compartment of a 'zero order' trough [24] of the KSV 2200 Barostat equipment (KSV Helsinki). The inhibitory power (*Z*) was calculated for each compound (Table 1) as described by Rasnac *et al.* [23].

RESULTS AND DISCUSSION

The initial screening of the present series of lipid mimetics as inhibitors of PLA2 has been based on a bulk radiometric assay. The findings, expressed as apparent IC$_{50}$ values are summarized in Table 1. The α-amino acid amides, **1, 5** appear to be more active that the α-amino acid esters **20** and **21** or the esters of the α-amino alcohols (**22, 23**). Comparison of the activities exhibited by the dipeptide analogues **24, 25, 26** and the corresponding alcohol **27** revealed the importance of the presence of a free

amino group. The functional groups seemed to influence the inhibitory activity in the order alcohol (**27**) > ester (**25**) > acid (**26**). Amides (**1–19**) and dipeptide analogues (**24, 25** bearing short (C5) and long (C14–C20) saturated and unsaturated side chains were designed to explore the specificity of the R1 and R2 groups. The 2-amino-hexanoic acid derivatives exhibited inhibitory activity in contrast to the α-amino hexadecanoic acid derivatives that were found to be inactive.

As expected, the tested compounds were found to inhibit the pancreatic PLA2 in a reversible manner. PLA2 was preincubated with the inhibitors for 30 min, at concentrations that had been observed to reduce the enzymatic activity significantly (70–90% inactivation). After preincubation, aliquots were removed and diluted 10–30 fold into the assay mixture (radioactive assay). The level of inhibition observed was decreased in respect to the dilution, corresponding to the values given by the dose response curves for each inhibitor. These findings suggested the reversibility of the observed inhibition [25]. In the case of irreversible inhibition, no alteration in the inhibitory activity should be expected after dilution with substrate.

Phospholipases are lipolytic enzymes that react at a lipid/water interface. Any molecule modifying the properties of the interface could affect the PLA2 activity [26]. The bulk assay system used for the initial evaluation of the compounds did not allow us to distinguish between true inhibitors acting directly on the active centre of the enzyme and the molecules – modifiers of the 'interfacial quality' that cause indirect 'inhibition' [26, 27]. The fact that most of the lipid mimetics that were found to be inactive during this study, stimulated the enzyme, has been attributed to the resemblance these compounds share with the natural lipids, and hence improve the 'interfacial quality' of the substrate used. This property could cast doubt about the validity of the findings regarding the true nature of the inhibition towards the PLA2.

The use of monolayer techniques for the study of phospholipase kinetics has been well documented [23]. The kinetic model developed by Ransac *et al.* [28] is applicable to water-insoluble molecules and therefore suitable for the study of the lipid mimetics. Mixed substrate/inhibitor monomolecular films at different mole fractions were used for the estimation of the inhibitory power (*Z*) of the tested compounds (Table 1).

The compounds exhibiting affinity for the enzyme greater than that of the substrate are expected to

have inhibitory power values greater than one, whereas for the inactive compounds the values are zero or negative. As can be seen from the results (Table 1) all the compounds that had no effect or stimulated PLA2, according to the results of the bulk assay, gave Z values close to 0. The compounds that had apparent IC_{50} values in the range of 10–30 μM gave Z values of 2–13. As can be seen from the results of Table 1, the results of the two assay systems are in agreement, and most of the conclusions drawn from the bulk assay are valid: the free amino group is important and there is preference for short (C4) and long (C16) side chains for the active α-amino acid amides. According to the results from the monolayer assay the dipeptide analogues **24–27** did not exhibit any significant inhibitory activity that could allow the confirmation of the initial conclusions concerning their differentiation.

The most potent compound of the studied series was the amide **1**, which gave an inhibitory power $Z = 13.3 + 0.7$. This value indicated a weak inhibition and is in the same range of potency as some of the acylamino analogues of phospholipids that have been designed and tested as PLA2 inhibitors with the same monolayer technique [22]. The inhibitory power of the lipid mimetics exhibited suggested that the active compounds inhibited the porcine pancreatic PLA2 by a direct interaction with the active centre and not through the modification of the quality of the interface.

The overall results of this study suggested that this type of lipid mimetic could contribute not only to the development of potent inhibitors of pancreatic PLA2 but also to other lipolytic enzymes as well, owing to their resemblance to the natural lipid substrates. The application of the monolayer technique for the evaluation of the inhibitors indicated their direct interaction with the enzyme and validated the results of this study. In comparing inhibition data obtained with the bulk and the monolayer methods one has to consider the huge difference in specific surfaces characterizing each system. As a consequence the phase partitioning of a water-soluble lipolytic enzyme between the interface and the water phase are also very different with both systems. This means that both experimental conditions are not directly comparable [22,23].

CONCLUSIONS

Further design and synthesis are currently underway in order to improve the exhibited inhibitory power and *in vivo* studies must be performed in order to evaluate the anti-inflammatory and other activities of the reported compounds.

Acknowledgements

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