Inhibition of Group IVA Cytosolic Phospholipase A_2 by Novel 2-Oxoamides in Vitro, in Cells, and in Vivo

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The Group IVA cytosolic phospholipase A_2 (GIVA PLA_2) is a particularly attractive target for drug development because it is the rate-limiting provider of proinflammatory mediators. We previously reported the discovery of novel 2-oxoamides that inhibit GIVA PLA_2 [Kokotos, G.; et al. *J. Med. Chem.* **2002**, *45*, 2891−2893]. In the present work, we have further explored this class of inhibitors and found that the 2-oxoamide functionality is more potent when it contains a long 2-oxoacyl residue and a free carboxy group. Long-chain 2-oxoamides based on γ-aminobutyric acid and γ-norleucine are potent inhibitors of GIVA PLA_2. Such inhibitors act through a fast and reversible mode of inhibition in vitro, are able to block the production of arachidonic acid and prostaglandin E_2 in cells, and demonstrate potent in vivo anti-inflammatory and analgesic activity.

Introduction

Phospholipase A_2 (PLA_2) catalyzes the hydrolysis of the *sn*-2 or middle fatty acyl chain from a phospholipid. The PLA_2 superfamily of enzymes consists of two large branches: the histidine and serine PLA_2 branches.[1] The histidine PLA_2's are similar small, secreted, Ca^{2+}-dependent enzymes that utilize a nucleophilic water molecule activated by the active site Ca^{2+}, aspartates, and especially a catalytic histidine (Groups I−III, V, IX−XIV). The other branch of the superfamily is composed of larger, typically cytosolic enzymes that utilize a nucleophilic serine (Groups IV and VI−VIII). Groups VII and VIII PLA_2 are also known as platelet activating factor (PAF) acetylhydrolases and utilize their nucleophilic serine as part of a typical catalytic triad of serine−histidine−aspartate. In the case of Group IVA PLA_2 (also referred to as the 85 kDa, cytosolic PLA_2), the nucleophilic serine is in a novel catalytic dyad involving only a serine and an aspartate.[2,3] The Group VI PLA_2 appears to utilize a similar catalytic mechanism[4] and is indeed inhibited by many compounds that inhibit the Group IVA PLA_2.[5−8]

The Group IVA PLA_2 (GIVA PLA_2) is a particularly attractive target for drug development because it is the rate-limiting provider of free polyunsaturated fatty acids and lysophospholipids that go on to form the eicosanoids and PAF, respectively. This information has been gathered through the use of many nonspecific and imperfect inhibitors in cellular and animal studies over the years. But perhaps the most compelling evidence for the central role of GIVA PLA_2 comes from the gene-targeted mice that lack GIVA PLA_2.[9−14] Various studies have found that these mice are much less prone to inflammatory pathological responses to disease, stresses, and physical injuries, which in essence protects them from cellular and systemic damage.[9−14] Furthermore, the mice lacking GIVA PLA_2 are fairly normal in all other respects.[9−11,13,14] The female null mice have some fertility problems mostly associated with their lack of labor-inducing prostaglandins and thus must be surgically delivered, but their pups are otherwise normal.[9,11,15] Therefore, these data suggest that synthetic inhibitors of GIVA PLA_2 would be exceedingly valuable for prevention and treatment of various inflammatory conditions and would avoid many side effects and drawbacks of current therapies and treatments.[16] Recently we discovered novel 2-oxoamides that inhibit GIVA PLA_2.[17] In the present work, we demonstrate that synthetic 2-oxoamides constitute a novel class of human GIVA PLA_2 inhibitors with promising anti-inflammatory and analgesic activity.

Rational Design of GIVA PLA_2 Inhibitors

A decade ago, a trifluoromethyl ketone analogue of arachidonic acid (AA) in which the COOH group was replaced by $COCF_3$ (AATFK, **1**, Scheme 1) was prepared and found to be a tight- and slow-binding inhibitor of the 85 kDa cytosolic human GIVA PLA_2.[18] As demonstrated with NMR studies, the inhibitor was bound to the enzyme's active site in a reversible manner and was in a slow exchange with the free ligand.[19] Another AA derivative, methylarachidonoyl fluorophosphonate

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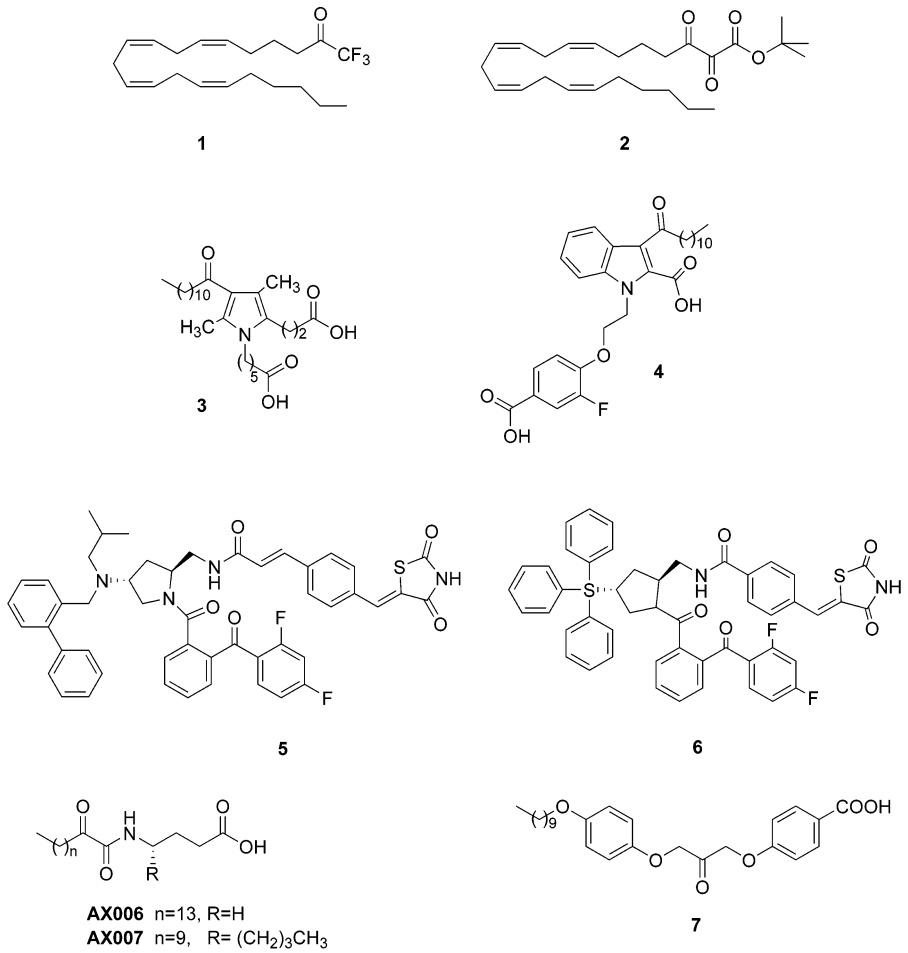
Scheme 1. Structures of Known Reversible GIVA PLA_2 Inhibitors

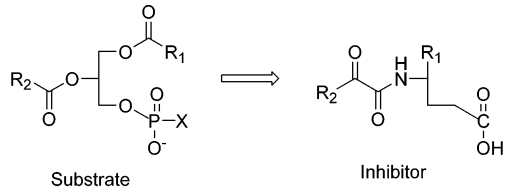


(MAFP), has been shown to irreversibly inactivate GIVA PLA_2.[20] The inhibition of GIVA PLA_2, in comparison with GVI PLA_2, by unsaturated and saturated fatty acid tricarbonyls[21] (**2**) and trifluoromethyl ketones[8] has been studied. Lehr has reported that (4-acylpyrrol-2-yl)-alkanoic acids[23] such as **3**, and 3-acyl 3-dodecanoylindol-2-carboxylic acids[24] such as **4** inhibit the cytosolic PLA_2 of platelets as measured by the blocking of AA release induced by the calcium ionophore A23187. Seno et al. have shown that pyrrolidine derivatives,[25] having the thiazolidinedione group at the end of the substitutent in the 2-position of the pyrrolidine ring and the 2-benzoyl benzoyl group at the 1-position (for example, compound **5**) are potent inhibitors of human GIVA PLA_2. An extension of this research led to the potent inhibitor pyrrophenone (**6**).[26] Despite these advances, potent and specific reversible competitive inhibitors of GIVA PLA_2 are not commercially available.

Novel inhibitors of GIVA PLA_2 may be rationally designed, taking into consideration the mechanism of the enzyme's catalytic action and the structure of the substrate. GIVA PLA_2 is an enzyme that acts at the lipid−water interface. Its catalytic mechanism proceeds through a serine−acyl intermediate using Ser-228 as the nucleophilic residue. The X-ray crystal structure of GIVA PLA_2 indicates that the enzyme consists of an N-terminal calcium-dependent lipid-binding C2 domain and a catalytic α/β hydrolase domain whose topology is distinct from that of other lipases.[3] GIVA PLA_2 possesses an unusual catalytic Ser-Asp dyad located in a deep cleft at the center of a hydrophobic funnel of the catalytic domain.[3]

Our strategy for the rational design of novel GIVA PLA_2 inhibitors is based on the principle that the inhibitor should consist of several parts: an electrophilic functionality (activated carbonyl group), which may react with the active-site serine; a lipophilic segment; and the appropriate chemical motifs necessary for specific interactions and proper orientation in the enzyme's active site. It has been demonstrated that lipophilic 2-oxoamides[27] and 2-oxoamide and bis-2-oxoamide triacylglycerol analogues[28] are efficient inhibitors of pancreatic and gastric lipases, enzymes containing a classical catalytic triad (Ser-His-Asp). Thus, among the various existing functionalities capable of reacting with the active-site serine, the 2-oxoamide functionality was chosen as the reactive group. The GIVA PLA_2 residue Arg-200 has been proposed to interact with the negative charge of its substrate phospholipids,[3] and therefore, an analogous carboxy group was incorporated into the 2-oxoamide backbone (Scheme 2). Such a design led to the discovery of the lead 2-oxoamide structures AX006 and AX007,[17] explored in the present work in detail. In the same year, another type of GIVA PLA_2 inhibitor based on a 1,3-disubstituted propan-2-one skeleton (**7**) was reported by Connolly et al.[29]

Scheme 2. Design of 2-Oxoamide Inhibitors



Synthesis of 2-Oxoamide Inhibitors

For the synthesis of inhibitors containing γ-aminobutyric acid (GABA), methyl 4-aminobutyrate (**9**) was coupled with 2-hydroxycarboxylic acids **8a**,**b** using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (WSCI)[30] as a condensing agent in the presence of 1-hydroxybenzotriazole (HOBt) (Scheme 3). Alkaline hydrolysis of **10a**,**b**, followed by oxidation with NaOCl in the presence of a catalytic amount of 4-acetamido-2,2,6,6-tetramethylpiperidine-1-yloxy free radical (AcNH-TEMPO)[31] produced compounds AX005 and AX006. GABA derivatives AX010 and AX011 were also synthesized (Scheme 3).

N-protected amino alcohols **11a**−**d** (Scheme 4) were prepared from the corresponding natural α-amino acids by the mixed-anhydride/$NaBH_4$[32] or the acyl fluoride/$NaBH_4$ method.[33] Alcohols **11a**−**d** were oxidized to the corresponding aldehydes **12a**−**d** by the NaOCl/AcNH-TEMPO method (Scheme 4), and the aldehydes were directly used in the next step without any purification.

There are three routes for the synthesis of γ-amino acid-based 2-oxoamide inhibitors depending on the ylide and the N protecting group used (Scheme 4). Wittig reactions of Boc-protected amino aldehydes **12a**−**c** with benzyl or methyl (triphenylphosphoranylidene)acetate led to α,β-unsaturated esters **13** and **14a**−**c**. The Boc group of **13** was removed, and the amino component was coupled with 2-hydroxydodecanoic acid by the WSCI/HOBt method. In a similar manner, **14a**−**c**, after hydrogenation, were converted into **17a**−**e**. Catalytic hydrogenation of **16** and saponification of **17a**−**e**, followed by oxidation with either pyridinium dichromate (PDC) or NaOCl/AcNH-TEMPO, led to the target compounds AX007, AX012, AX013, AX016, AX017, and AX024. γ-Proline-based inhibitors, AX018 and AX019, and AX008, the enantiomer of AX007, were also prepared. The Z-protected amino aldehyde (**12d**) was treated with *tert*-butyl (triphenylphosphoranylidene)-acetate to give compound **15**. After catalytic hydrogenation, the amino component was coupled with 2-hydroxypentadecanoic acid by the WSCI/HOBt method. Oxidation of **18**, followed by the removal of the *tert*-butyl group with CF_3COOH, led to AX014.

The synthesis of γ-serine-based inhibitors is depicted in Scheme 5. Boc-L-Ser-OH (**19**) was reacted with a medium-chain alkyl iodide and was converted into serinol derivative **21**. The α,β-unsaturated ester **22** was prepared by oxidation and reaction with benzyl (triphenylphosphoranylidene)acetate. After removal of the Boc group, coupling with 2-hydroxydodecanoic acid, hydrogenation, and oxidation, 2-oxoamide derivative AX027 was obtained. Its enantiomer, AX026, was prepared in a similar manner.

AX001-AX004, AX009, and AX015 (see Table 1 for structures) were prepared as described.[27b]

In Vitro Data for AX006 and AX007

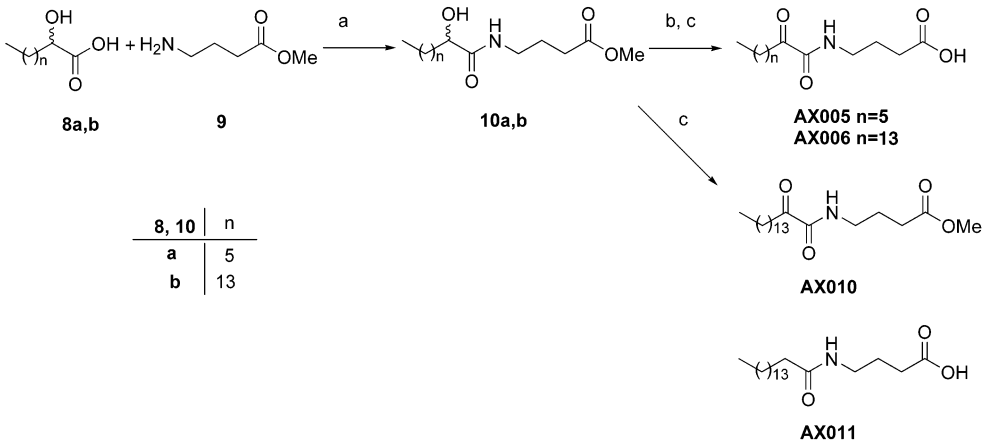
All the inhibitors synthesized were tested against GIVA PLA_2, and the results of inhibition are presented in Table 1 using either percent inhibition or $X_I(50)$. The $X_I(50)$ is the mole fraction of inhibitor in the total substrate interface required to inhibit the enzyme by 50%. The reason that $X_I(50)$ is used instead of the more common IC_{50} or K_I is that GIVA PLA_2 is active only at phospholipid surfaces such as cell membranes, phospholipid vesicles, and phospholipid micelles, where its substrate phospholipids reside. Almost all inhibitors of PLA_2's partition at least to some degree into the phospholipid surface because they usually have a hydrophobic portion that complements the hydrophobic active site of the PLA_2. When these inhibitors partition into the surface, an important physical effect called surface dilution comes into play. In this case, the affinity of the PLA_2 for the inhibitor depends not on the three-dimensional (bulk) concentration of the inhibitor in molar units but rather on the two-dimensional (surface) concentration of the inhibitor in mole fraction units. For these reasons, IC_{50} values obtained in different assay systems (even as minor changes as varied substrate concentrations) cannot be compared.

Primary 2-oxoamides (AX001 and AX015) containing either a long, linear chain or a phenyl-substituted chain do not inhibit GIVA PLA_2. Secondary 2-oxoamides lacking a carboxylic acid functionality (AX002, AX003, AX004, and AX009) also failed to inhibit the enzyme. However, 2-oxoamides based on GABA and γ-norleucine (AX006, AX007, and AX008) do inhibit GIVA PLA_2.

Figure 1 shows the data used to determine the $X_I(50)$ values for entries 6, 7, and 8,[17] now named AX006, AX007, and AX008, respectively. The data for AX006 and AX007 were easily fit to logarithmic functions using nonlinear least-squares fits to determine the empirical $X_I(50)$ values. This fit is useful for obtaining $X_I(50)$ values with error values and does not represent a fit to any kinetic equations. The data for AX008 clearly do not fit a logarithmic function but rather are linear. Therefore, the data for AX008 were fit using a linear least-squares fit. AX007 and AX008 are enantiomeric, so the 8-fold difference in their $X_I(50)$ values and the shape of their inhibition curve provide important insights into the GIVA PLA_2 active site. In particular, all potent inhibitors fit logarithmic functions while poor inhibitors fit linear functions. It seems that the chirality introduced by a hydrophobic side chain adjacent to the 2-oxoamide nitrogen in a γ-acid backbone provides stereospecific enhancements of the binding interaction.

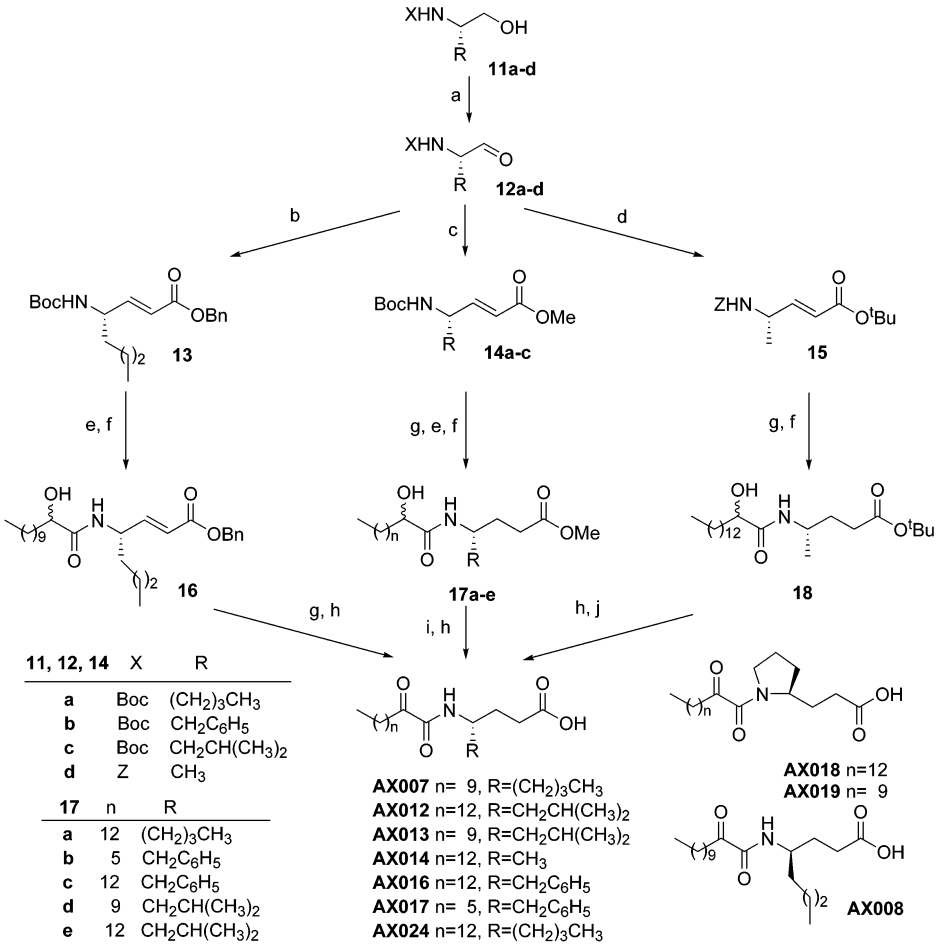
The in vitro activity assays used to determine the potency of the various 2-oxoamide compounds were done in the presence of 0.006 mole fraction phosphatidylinositol 4,5-bisphosphate (PIP_2). This is a tight binding activator of GIVA PLA_2.[34,35] Without PIP_2 in these particular substrate micelles, GIVA PLA_2 activity drops to almost undetectable levels. Therefore, it was important to rule out any interference with the GIVA PLA_2−PIP_2 interaction by the 2-oxoamide inhibitors. We have tested this using an alternative substrate micelle that allows the measurement of GIVA PLA_2 activity without any PIP_2 present, as described in the Supporting Information of our initial study,[17] consisting of 1-palmi-

Scheme 3. Synthesis of GABA-Based Inhibitors[a]



[a] Reagents and conditions: (a) WSCI, Et_3N, HOBt, CH_2Cl_2; (b) 1 N NaOH, dioxane/H_2O 9:1; (c) NaOCl, AcNH-TEMPO, NaBr, $NaHCO_3$, EtOAc/$PhCH_3$/H_2O 3:3:0.5, −7 °C.

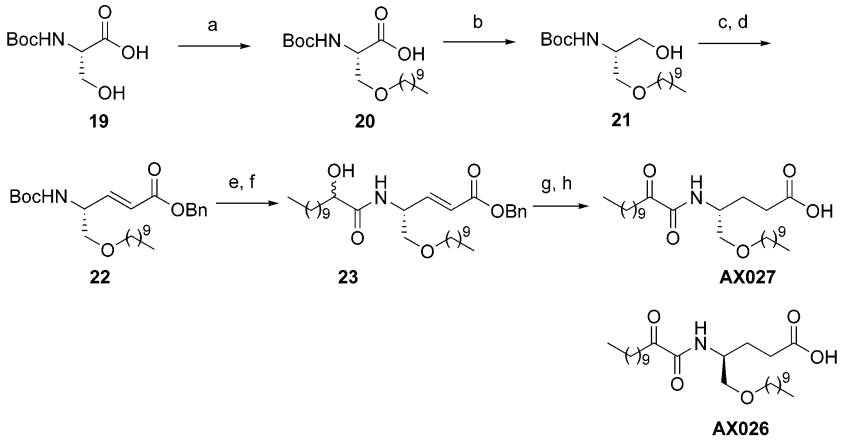
Scheme 4. Synthesis of γ-Amino Acid Based Inhibitors[a]



[a] Reagents and conditions: (a) NaOCl, AcNH-TEMPO, NaBr, $NaHCO_3$, EtOAc/$PhCH_3$/H_2O 3:3:0.5, −5 °C; (b) $Ph_3P{=}CHCOOBn$, THF, reflux, 1 h; (c) $Ph_3P{=}CHCOOMe$, THF, reflux, 1 h; (d) $Ph_3P{=}CHCOO^tBu$, THF, reflux, 1 h; (e) 4 N HCl/THF; (f) $CH_3(CH_2)_nCHOHCOOH$, WSCI, HOBt, Et_3N, CH_2Cl_2; (g) H_2, 10% Pd/C; (h) NaOCl, AcNH-TEMPO, NaBr, $NaHCO_3$, EtOAc/$PhCH_3$/H_2O 3:3:0.5, 0 °C, then HCl; (i) 1 N NaOH, dioxane/H_2O 9:1, then HCl; (j) 50% CF_3COOH, CH_2Cl_2.

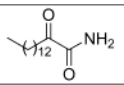
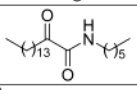
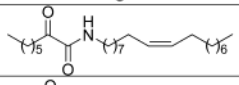
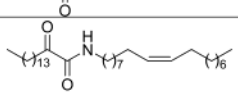
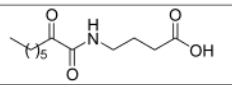
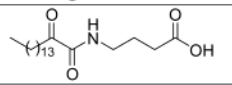
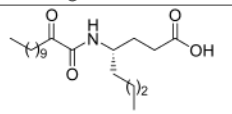
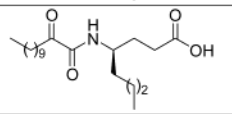
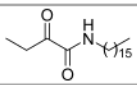
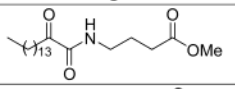
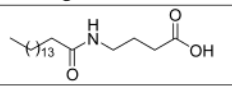
toyl-2-arachidonoylphosphatidylcholine (PAPC) and Triton X-100 (TX-100) at a 1 mM to 3 mM ratio. As shown in Figure 2, the presence of 0.01 mole fraction of AX006 in these micelles that lack PIP_2 does indeed inhibit GIVA PLA_2 by about 50%. This is consistent with a true inhibition of GIVA PLA_2 by AX006 in the PIP_2-containing micelles. We also tested AX007 in conditions that lacked PIP_2. Figure 3 shows the dose-dependent inhibition of GIVA PLA_2 by AX007. As in Figure 1, the inhibition data have been fit to a logarithmic function. The $X_I(50)$ derived from the equation of the fit was 0.0027 ± 0.0012 mole fraction. This is the same magnitude as the $X_I(50)$ determined in the presence of PIP_2, indicating a true inhibition of GIVA PLA_2 independent of PIP_2. This also compares favorably with the $X_I(50)$ reported for pyrrophenone (0.002 mole fraction).[26]

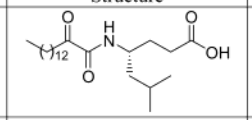
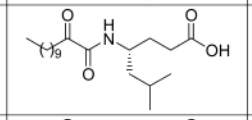
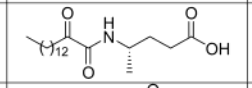
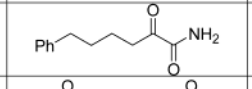
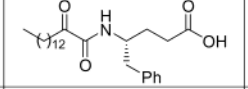
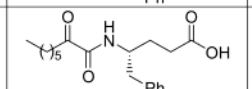
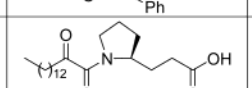
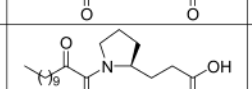
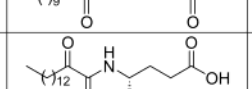
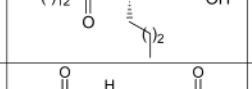
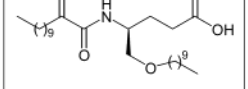
Scheme 5. Synthesis of γ-serine Based Inhibitors[a]



[a] Reagents and conditions: (a) NaH, $C_{10}H_{21}I$, DMF, 0 °C; (b) ClCOOEt, NMM, THF, −10 °C, then $NaBH_4$, MeOH; (c) NaOCl, AcNH-TEMPO, NaBr, $NaHCO_3$, EtOAc/$PhCH_3$/H_2O 3:3:0.5, −5 °C; (d) Ph_3P=CHCOOBn, THF, reflux, 1 h; (e) 4 N HCl/THF; (f) $CH_3(CH_2)_9CHOHCOOH$, WSCI, HOBt, Et_3N, CH_2Cl_2; (g) H_2, 10% Pd/C; (h) NaOCl, AcNH-TEMPO, NaBr, $NaHCO_3$, EtOAc/$PhCH_3$/H_2O 3:3:0.5, 0 °C, then HCl.

Table 1. In Vitro Inhibition of GIVA PLA_2 by 2-Oxoamides

Entry[a]	Structure	X_i(50) (mole fraction)	Highest Conc. (mole fraction) w/% Inhibition	ClogP
AX001		N.D.[b]	0.01; 0%	4.2
AX002		N.D.	0.02; 22%	9.0
AX003		N.D.	0.01; 0%	10.6
AX004		N.D.	0.05; 0%	14.8
AX005		N.D.	0.05; 0%	2.4
AX006		0.017 ± 0.009		6.6
AX007		0.009 ± 0.004		6.4
AX008		0.068 ± 0.005		6.4
AX009		N.D.	0.01; 0%	7.9
AX010		N.D.	0.08; 0%	7.1
AX011		N.D.	0.08; 22%	6.2

Entry	Structure	X_i(50)	Inhibition	ClogP
AX012		0.017 ± 0.006		6.8
AX013		0.021 ± 0.007		5.8
AX014		0.021 ± 0.006		6.4
AX015		N.D.	0.048, 0%	0.8
AX016		0.035 ± 0.016		7.1
AX017		N.D.	0.091; 24%	3.4
AX018		N.D.	0.091; 24%	5.7
AX019		N.D.	0.091; 0%	4.0
AX024		0.010 ± 0.004		8.0
AX026		0.045 ± 0.007		9.2
AX027		0.047 ± 0.006		9.2

[a] AX001-AX008 are entries 1−8 from Kokotos et al. *J. Med. Chem.* **2002**, *45*, 2891−2893. [b] Not determinable.

Various inhibitors of GIVA PLA_2 have been shown to be slow-binding and only slowly reversible. AATFK and MAFP inhibit GIVA PLA_2 in both concentration- and time-dependent manners in the presence and absence of a substrate interface.[5−8] To test the type of inhibition by the 2-oxoamide inhibitors, AX006 was preincubated

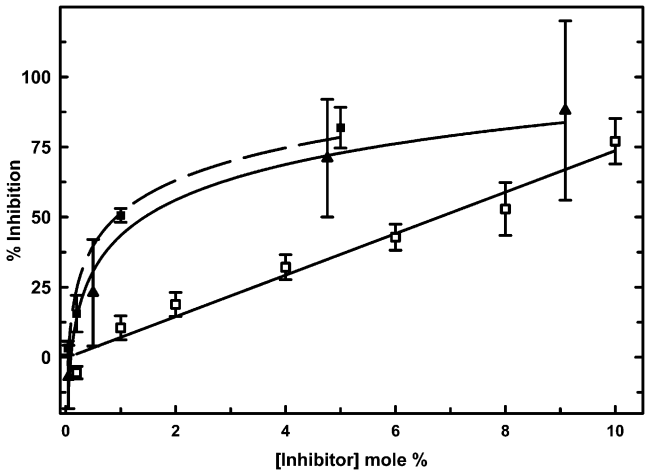


Figure 1. Dose−response curves for 2-oxoamide inhibitors. The activity of GIVA PLA_2 was tested on mixed micelles composed of TX-100 (400 μM), 1-palmitoyl-2-(1-[^{14}C]-arachidonoyl) PC (97 μM, 100 000 cpm), and PIP_2 (3 μM, 0.6 mol %). The surface concentration of various inhibitors in the mixed micelles was increased as shown for AX006 (▲), AX007 (■), and AX008 (□). The data for AX006 and AX007 were fit to a simple logarithmic function (AX006 (solid curve) and AX007 (dashed curve)). The $X_I(50)$ determined by this fit was 0.017 ± 0.009 for AX006 and 0.009 ± 0.004 for AX007. The data for AX008 were fit to a linear function. The $X_I(50)$ for AX008 determined by this fit was 0.068 ± 0.005.

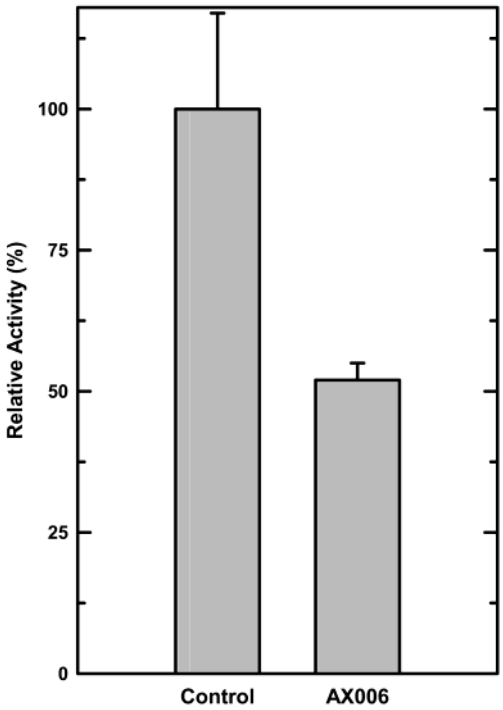


Figure 2. Inhibition of GIVA PLA_2 by AX006 is independent of PIP_2. The relative activity of GIVA PLA_2 was tested on mixed micelles composed of TX-100 (3 mM) and 1-palmitoyl-2-(1-[^{14}C]-arachidonoyl) PC (1 mM, 200,000 cpm) with no PIP_2 present. The surface concentration of AX006 was 0 mole fraction (left bar) and 0.01 mole fraction (40 μM, right bar). The data for both bars have been normalized to the control (left bar).

with GIVA PLA_2 without substrate for varying lengths of time, after which the mix was added to the assay solution (Figure 4A). The resulting activity assay showed that there was no increase in inhibition with preincubation times up to 30 min. This indicated a fast-binding

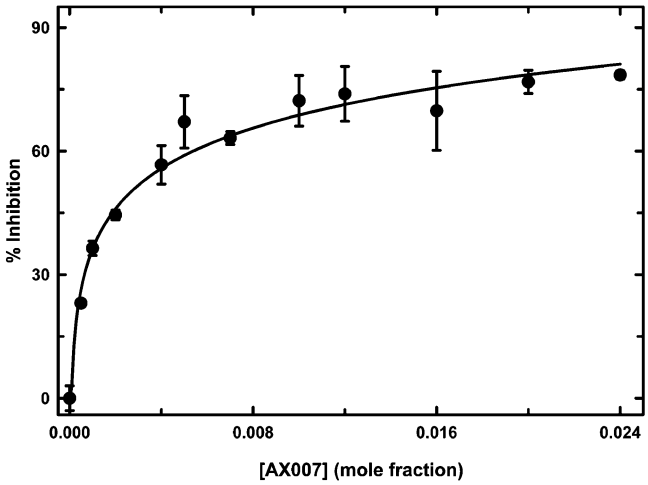


Figure 3. Inhibition of GIVA PLA_2 by AX007 is independent of PIP_2. The activity of GIVA PLA_2 was tested on mixed micelles composed of TX-100 (3 mM) and 1-palmitoyl-2-(1-[^{14}C]-arachidonoyl) PC (1 mM, 200 000 cpm) with no PIP_2 present. The surface concentration of AX007 was increased as shown. The curve is a fit of the data to a simple log function. The curve values give an $X_I(50)$ of 0.0027 ± 0.0012 mole fraction.

mode of inhibition of AX006. The concentration of AX006 for preincubation was 200 μM but dropped to 20 μM (0.005 mole fraction) after dilution of the enzyme−inhibitor mix into the assay solution. The assay results show that preincubation at a higher concentration followed by dilution did not yield any increased inhibition, suggesting that AX006 is a completely reversible inhibitor.

We also tested the more potent, chiral AX007 to see if it were a fast, reversible inhibitor of GIVA PLA_2. We preincubated GIVA PLA_2 with 20 μM AX007 and then diluted the preincubated GIVA PLA_2 into the assay solution that also contained 20 μM AX007. As shown in Figure 4B, a 4 h preincubation did not change the level of inhibition of AX007. This indicates that there is no time-dependent increase in inhibition of GIVA PLA_2 by AX007. In contrast, Figure 4C shows the results when AX007 was present at 20 μM during the preincubation and was then diluted 10-fold into the assay mix. This dilution brought the final AX007 concentration down to 2 μM (0.0005 mole fraction) and resulted in a significant reduction in the inhibition of GIVA PLA_2 by AX007 (Figure 4C). The significantly reduced inhibition by the diluted AX007 (at 0.0005 mole fraction) is consistent with the results shown in Figure 1. Together, the results for AX006 and AX007 strongly indicate that 2-oxoamide inhibitors of GIVA PLA_2 function through a fast and fully reversible mode of inhibition.

Structure−Activity Relationships

The discovery that the (*S*)-γ-norleucine-containing 2-oxoamide (AX007) enhances inhibitory potency in an enantioselective fashion (8-fold more potent than (*R*)-AX008 and 2-fold more potent than nonchiral AX006) led to the further exploration of the nature of the (*S*) side chain. Substitutions of side chains at the (*S*)-γ-position revealed that short hydrophobic side chains were preferred. Two leucine variants, AX012 and AX013, and an alanine variant, AX014, were as potent as AX006 but only about half as potent as the norleucine-contain-

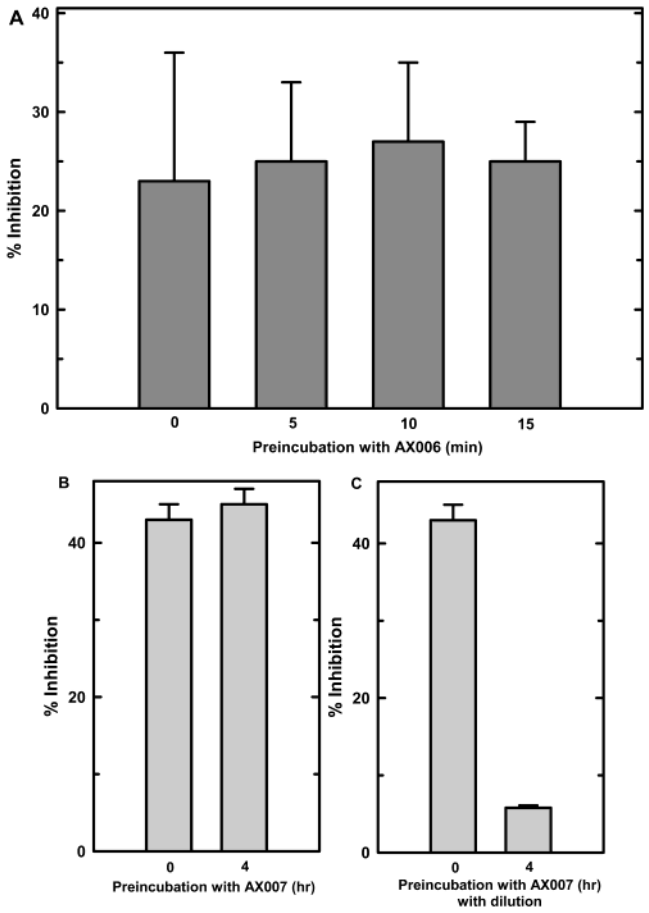


Figure 4. Immediate and reversible inhibition of GIVA PLA_2 by AX006 and AX007. The activity of GIVA PLA_2 was tested on mixed micelles composed of TX-100 (400 μM), 1-palmitoyl-2-(1-[^{14}C]-arachidonoyl) PC (97 μM, 100 000 cpm), and PIP_2 (3 μM, 0.6 mol %). (A) The micelles also contained a final concentration of 0.005 mole fraction of AX006 (20 μM). GIVA PLA_2 was first preincubated with AX006 for the indicated times (at 200 μM) before 10-fold dilution of the enzyme and inhibitor into the substrate-containing solution. (B) The micelles also contained a final concentration of 0.005 mole fraction of AX007 (20 μM). GIVA PLA_2 was first preincubated with AX007 (20 μM) for 0 or 4 h before it was added to the substrate-containing solution that also contained AX007 (20 μM). (C) The micelles also contained a final concentration of 0.005 mole fraction of AX007 (20 μM). GIVA PLA_2 was first preincubated with AX007 (20 μM) for 0 or 4 h before it was added to the substrate-containing solution that contained no additional AX007. This resulted in a final concentration for AX007 of 0.0005 mole fraction (2 μM).

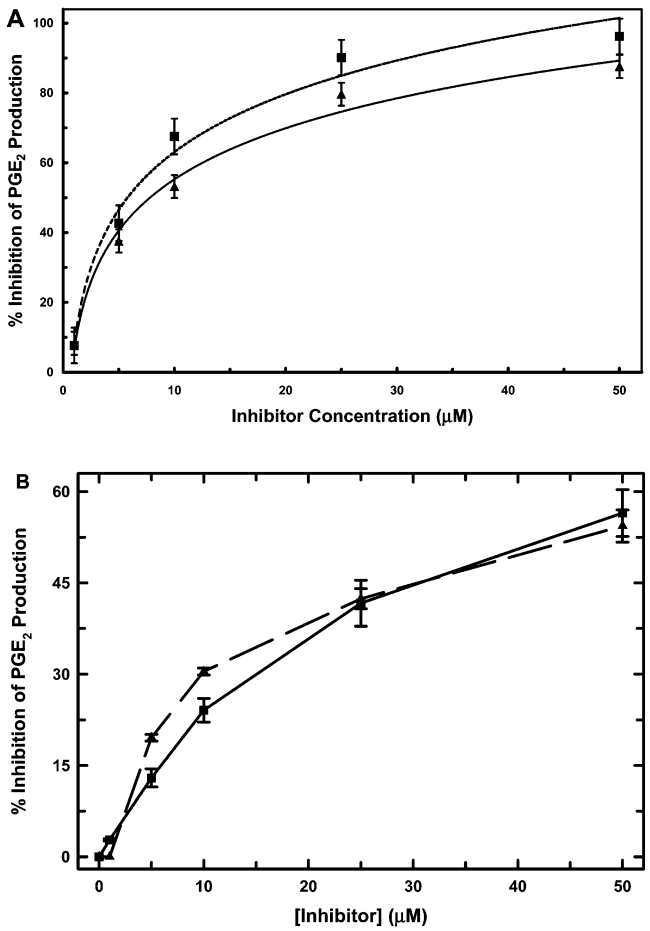


Figure 5. Inhibition of cellular PGE_2 production by AX006 and AX007. Varying concentrations of AX006 (▲) or AX007 (■) were added to $P388D_1$ cells 30 min. prior to stimulation. (A) The cells were stimulated with LPS for 18 h as described. After stimulation, the media was harvested and the PGE_2 levels in the media were quantitated as described. The data were normalized to control DMSO-treated LPS-stimulated cells to appear as percent inhibition. The lines correspond to a nonlinear least-squares fit of the data to logarithmic functions (AX006 (solid curve) and AX007 (dashed curve)). These functions were then used to calculate the IC_{50} for AX006 (7.8 μM) and AX007 (5.8 μM). (B) The cells were stimulated with LPS for 1 h and PAF for 15 min as described. After stimulation, the media was harvested and the PGE_2 levels in the media were quantitated as described. The data were normalized to control DMSO-treated LPS/PAF stimulated cells to appear as percent inhibition. The data points are connected by a dashed line (AX006) and a solid line (AX007) for ease of visualization.

ing AX007. Two phenylalanine variants (AX016 and AX017) and two proline variants (AX018 and AX019) were significantly less potent. AX016 was nearly 4-fold less potent than AX007, while AX017 was more than 10-fold less potent. AX018 was also more than 10-fold less potent than AX007, while AX019 had no detectible inhibition at the highest concentration tested. Furthermore, the substitution of serine-based, long aliphatic ether side chains (AX026 and AX027) led to poor GIVA PLA_2 inhibitors. These enantiomeric compounds exhibited essentially identical linear modes of inhibition typical of poor inhibitors (data not shown) and showed no stereoselectivity. These results together indicate that the inhibitory potency and shape of the inhibitory profile relate to the ability of the 2-oxoamide's short, hydrophobic side chain to bind properly in a stereoselective manner to the GIVA PLA_2 active site.

The role of the free carboxy group was shown to be important in our preliminary work by comparing AX006 and AX002.[17] We have further confirmed the need for the negative charge by testing a variant of AX006 that contained a carboxymethyl ester (AX010) in place of the free carboxy group (AX006). AX010 had no measurable inhibition up to 0.08 mole fraction, whereas AX006 almost completely inhibited GIVA PLA_2 at this concentration. The carboxylic acid group in the 2-oxoamide compounds is a shared feature among some previously identified inhibitors of the small, secreted histidine PLA_2[36] as well as other GIVA PLA_2 inhibitors, compounds **3**, **4**, and **7** (Scheme 1).[23,24,29] The role of the

2-oxoamide functionality becomes obvious in a comparison of the effective GIVA PLA_2 inhibitor AX006 (2-oxoamide) with AX011 (corresponding simple amide), which barely inhibits GIVA PLA_2.

Lipophilicity is a very important parameter in the case of GIVA PLA_2 inhibitors because this enzyme exhibits its catalytic action at the lipid−water interface. From the values of ClogP (Table 1) calculated for the inhibitors by CQSAR, version 4.30 (Biobyte Corp.), it seems that the ClogP values of the active compounds fall into the range 5−8. The effect of the lipophilicity on activity is obvious upon comparison of the active AX006 (ClogP = 6.6) with the inactive compound AX005 (ClogP = 2.4). However, the lipophilicity by itself is not sufficient for the expression of activity (see, for example, AX002 and AX004). Major effects related to hydrophobicity are not seen for most of the inhibitors, since the ClogP values for similarly potent inhibitors vary from 5.8 (AX013) to 8.0 (AX024), suggesting complete partitioning over this range. It should be noted that a high ClogP (>8) renders an inhibitor an unlikely candidate for pharmaceutical purposes. For comparison, the ClogP values of other recent lead GIVA PLA_2 inhibitors are 9.6 for pyrrophenone[26] and 7.85 for the lead propane-2-one-based inhibitor.[29]

By varying the 2-oxoacyl chain length, we have explored the differences in inhibition based on hydrophobicity. Using phenylalanine variants, we looked specifically at the saturated chain length of the 2-oxoacyl chain (13 carbons for AX016). When the length was shortened to six carbons (AX017), the potency went down at least 4-fold. This same effect was seen in the preliminary study comparing AX005 and AX006 that differed by eight carbons.[17] Smaller changes in the chain length for already hydrophobic compounds can be seen to have smaller or negligible effects. This is seen by comparing the γ-norleucine-containing AX007 (10-carbon chain) and AX024 (13-carbon chain), which have equivalent inhibitory potency. Likewise, the γ-leucine-containing AX013 (10 carbons) and AX012 (13 carbons) have a nearly equivalent inhibitory potency. It is possible that partitioning to the micelles is affected by the less hydrophobic shortened acyl chains (such as AX005), but it is also possible that the shorter-chained compounds could have a weaker interaction with the deep hydrophobic active site of GIVA PLA_2. By analogy to phospholipid substrate, it is likely that this hydrophobic chain occupies part of the substrate (AA) binding pocket.

Lehr[23] and Connolly et al.[29] have proposed models for the interaction of cytosolic PLA_2 with indole and pyrrole inhibitors and 1,3-disubstituted propan-2-one based inhibitors. According to the findings of this research, we propose a four-point model for the binding of inhibitors to GIVA PLA_2, as shown in Figure 6. The enzyme−inhibitor complex is likely to be stabilized by the "oxyanion hole" corresponding to Gly 197, 198, and 229 within the enzyme active site. The lipophilic long carbon chain of the inhibitor may be accommodated in the hydrophobic binding site, where the *sn*-2 arachidonoyl residue of the natural substrate binds. The carboxy group may bind to the proposed polar binding site (Arg 200) in a manner similar to that of the phosphate group of phospholipids. In addition, the results of this work indicate the presence of an important fourth binding site that interacts with the side chain adjacent to the amide. This side chain binding site exhibits enantioselectivity and seems to accommodate a short, preferably linear, carbon chain. This part of the inhibitor may correspond to the *sn*-1 acyl chain of the natural substrate phospholipid. Alternatively, because inhibition is higher with shorter side chains than with a typical *sn*-1 acyl chain, it is possible that the side chain is fitting into a small pocket of GIVA PLA_2 not exploited in substrate binding.

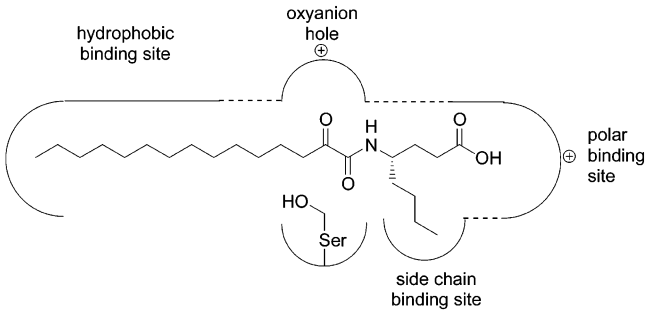


Figure 6. Model for the binding mode of 2-oxoamide inhibitors in the active-site crevice of GIVA PLA_2.

Table 2. Ex Vivo and in Vivo Activity of Selected 2-Oxoamide Inhibitors

	$P388D_1$ long-term LPS IC_{50} (μM)		rat in vivo	
entry	AA-derived products	PGE_2 production	anti-inflammatory activity ED_{50} (mmol/kg)[a]	analgesic activity[b] (%)
AX006	8.6 ± 3.5	7.8 ± 1.8	0.01	93
AX007	7.6 ± 2.4	5.8 ± 2.1	0.1	63
AX012	4.6 ± 1.5	10 ± 5	0.008	69

[a] Statistical significance of results was established using the Student's *t*-test, $P < 0.001$. [b] Analgesic activity (% analgesis) for the corresponding ED_{50} doses.

Ex Vivo Data: Stimulation of the $P388D_1$ Murine Macrophage Cell Line

AX006, AX007, and AX012 were tested on the murine $P388D_1$ macrophage-like cell line for inhibition of the GIVA PLA_2 in the long-term lipopolysaccharide (LPS) stimulation pathway (Table 2). GIVA PLA_2 is the central enzyme for the long-term LPS pathway through its ability to produce AA, which subsequently can be made directly into PGE_2.[37] The AA can also be used to up-regulate the message and protein levels of Group V PLA_2, which in turn produces its own AA that goes on to form PGE_2.[38,39] Blocking GIVA PLA_2 with 2-oxoamide inhibitors potently inhibited the release of AA with IC_{50} values in the low micromolar range (Table 2). This inhibition is even more potent than that seen previously with 25 μM MAFP, which reached a maximal inhibition at about 60% inhibition.[38]

Previous results showed that the substantial, but not complete, inhibition of AA release by MAFP correlated with complete abrogation of PGE_2 production.[38] As shown in Figure 5A, both AX006 and AX007 were able to completely block prostaglandin E_2 (PGE_2) release with the addition of 50 μM inhibitor, and neither showed cytotoxicity at this dose. Moreover, the dose−response data nicely fit to logarithmic functions. The IC_{50} values for the inhibition of PGE_2 release derived from these fits were 7.8 μM for AX006 and 5.8 μM for AX007 (Figure 5 and Table 2). Since MAFP interferes with the

measurement of PGE_2 release,[38] the ability of GIVA PLA_2 inhibitors to completely block PGE_2 is shown here for the first time. Previous reports did show that PGE_2 production was totally blocked by the cyclooxygenase-2-specific inhibitor, NS-398, at 5 μM and the secreted PLA_2 inhibitor LY311727 at 25 μM.[38]

Additionally, both AX006 and AX007 were tested in the LPS/PAF stimulation conditions of the $P388D_1$ cells (Figure 5B). These activation conditions result in the release of AA from cellular membranes by GIVA PLA_2.[37] Both of these inhibitors showed essentially identical inhibition of the PGE_2 release from the cells. The inhibition reached close to 60% at the maximal dosage tested (50 μM). This is also consistent with previous results indicating that LPS/PAF stimulation produced PGE_2 by two separate pathways, through GIVA PLA_2 and the Group V PLA_2. MAFP and LY311727 have each been shown to partially block AA and PGE_2 production.[40] Thus, the data shown in Figure 5B indicate that AX006 and AX007 inhibit the $P388D_1$ GIVA PLA_2 under the LPS/PAF stimulation conditions, resulting in the expected partial abrogation of PGE_2 release. Together, the data in the $P388D_1$ cells confirm that the 2-oxoamide inhibitors AX006, AX007, and AX012 can get into the cells and target GIVA PLA_2 without any detectible cytotoxicity.

In Vivo Data

To evaluate the anti-inflammatory activity of 2-oxoamide inhibitors, the rat paw carrageenan-induced edema assay[41] was employed as a model for acute inflammation. Indomethacin was used in these experiments as a reference drug and gave 47% inhibition of inflammation at 0.01 mmol/kg. The acetic acid writhing test[42] was used to assess the analgesic activity in rats. Acetylsalicylate was used in these experiments as a reference drug and gave 93.4% analgesis at 1 mmol/kg. The results of in vivo anti-inflammatory and analgesic activity of selected 2-oxoamide inhibitors are presented in Table 2. AX006 and AX012 exhibit in vivo anti-inflammatory activities comparable to that of indomethacin, whereas AX007 presented a weaker effect. Additionally all three compounds also showed analgesic activity at the ED_{50} dosage determined by anti-inflammatory activity and were significantly more potent than acetylsalicylate. There is no obvious reason for the higher efficacy of AX006 and AX012 compared with AX007, but the bioavailability and physiological half-life will have to be considered.

Conclusion

In conclusion, we have developed a novel class of human GIVA PLA_2 inhibitors. Structure−activity relationship studies have revealed that potent GIVA PLA_2 inhibitors contain (a) the 2-oxoamide funtionality, (b) a free carboxy group at a distance corresponding to γ-amino acid, (c) a long 2-oxoacyl residue, and (d) a short, preferably linear, aliphatic side chain. As demonstrated by selected examples, 2-oxoamide compounds inhibit GIVA PLA_2 through a fast and reversible mode of inhibition and block the production of AA and PGE_2. Furthermore, they exhibit potent in vivo anti-inflammatory and analgesic activity. Therefore, 2-oxoamides are promising novel agents for the prevention and treatment of various inflammatory conditions.

Experimental Section

Melting points were determined on a Buchi 530 apparatus and are uncorrected. Specific rotations were measured at 25 °C on a Perkin-Elmer 343 polarimeter using a 10 cm cell. NMR spectra were recorded on a Varian Mercury (200 Mz) spectrometer. All amino acid derivatives were purchased from Fluka Chemical Co. TLC plates (silica gel 60 F_{254}) and silica gel 60 (70−230 or 230−400 mesh) for column chromatography were purchased from Merk. Visualization of spots was effected with UV light and/or phosphomolybdic acid and/or ninhydrin, both in EtOH stain. THF, toluene, and Et_2O were dried by standard procedures and stored over molecular sieves or Na. All other solvents and chemicals were reagent grade and used without further purification. All the products gave satisfactory elemental analysis results.

A. General Method for the Coupling of 2-Hydroxy Acids with Amino Components. To a stirred solution of 2-hydroxy acid (2.0 mmol) and the hydrochloride amino component (2.0 mmol) in CH_2Cl_2 (20 mL), Et_3N (6.2 mL, 4.4 mmol) and subsequently WSCI (0.42 g, 2.2 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 overnight at room temperature. The solvent was evaporated under reduced pressure, and EtOAc (20 mL) was added. The organic layer was washed consecutively with brine, 1 N HCl, brine, 5% $NaHCO_3$, and brine, dried over Na_2SO_4, and evaporated under reduced pressure. The residue was purified by column chromatography using $CHCl_3$ as the eluent.

B. General Method for Saponification of 2-Hydroxyamides Containing Ester Groups. To a stirred solution of a 2-hydroxyamide containing an ester group (2.00 mmol) in a mixture of dioxane−H_2O (9:1, 20 mL), 1 N NaOH (2.2 mL, 2.2 mmol) was added, and the mixture was stirred for 12 h at room temperature. The organic solvent was evaporated under reduced pressure, and H_2O (10 mL) was added. The aqueous layer was washed with EtOAc, acidified with 1 N HCl, and extracted with EtOAc (3 × 12 mL). The combined organic layers were washed with brine, dried over Na_2SO_4, and evaporated under reduced pressure. The residue was purified after recrystallization [EtOAc−petroleum ether (bp 40−60 °C)].

C. General Method for the Oxidation of 2-Hydroxyamides Containing Carboxylic Groups. To a solution of a 2-hydroxyamide (2.00 mmol) in a mixture of toluene−EtOAc (1:1, 12 mL), a solution of NaBr (0.22 g, 2.1 mmol) in H_2O (1 mL) was added, followed by AcNH-TEMPO (4 mg, 0.02 mmol). To the resulting biphasic system, which was cooled to 0 °C, an aqueous solution of 0.35 M NaOCl (6.2 mL, 2.2 mmol), containing $NaHCO_3$ (0.50 g, 6 mmol), was added dropwise with vigorous stirring at 0 °C over 1 h. The mixture was stirred for 15 min at 0 °C, and H_2O (4 mL) was added. The aqueous layer was separated, acidified with 1 N HCl, and extracted with EtOAc (2 × 12 mL). The combined organic layers were washed consecutively with 5% aqueous KI (12 mL), 10% aqueous $Na_2S_2O_3$ (12 mL), and brine, dried over Na_2SO_4, and evaporated under reduced pressure. The residue was purified by column chromatography using EtOAc as the eluent.

D. General Method for the Oxidation of 2-Hydroxyamides Containing Ester Groups. To a solution of a 2-hydroxyamide (5.00 mmol) in a mixture of toluene−EtOAc (30 mL), a solution of NaBr (0.54 g, 5.25 mmol) in water (2.5 mL) was added, followed by AcNH-TEMPO (11 mg, 0.050 mmol). To the resulting biphasic system, which was cooled at −5 °C, an aqueous solution of 0.35 M NaOCl (15.7 mL, 5.50 mmol) containing $NaHCO_3$ (1.26 g, 15 mmol) was added dropwise while stirring vigorously at −5 °C over a period of 1 h. After the mixture had been stirred for a further 15 min at 0 °C, EtOAc (30 mL) and H_2O (10 mL) were added. The aqueous layer was separated and washed with EtOAc (20 mL). The combined organic layers were washed consecutively with 5% aqueous citric acid (30 mL) containing KI (0.18 g), 10% aqueous $Na_2S_2O_3$ (30 mL), and brine and dried over Na_2SO_4.

The solvents were evaporated under reduced pressure, and the residue was purified by column chromatography [EtOAc−petroleum ether (bp 40−60 °C) 1:9].

E. General Method for the Oxidation of N-Protected Amino Alcohols to Aldehydes. Method D was followed, but the residue was used immediately in the next step without any purification.

F. Wittig Reaction of N-Protected α-Aminoaldehydes with Stabilized Ylides. To a solution of the N-protected α-aminoaldehyde **12a**−**d** (5.00 mmol) in dry THF (25 mL), Ph_3P=CHCOOX (*X* = Me, ^{t}Bu, Bn) (5.5 mmol) was added, and the reaction mixture was refluxed for 1 h. Saturated aqueous NH_4Cl (20 mL) was added and extracted with Et_2O (3 × 10 mL). The combined organic phases were washed with brine and dried (Na_2SO_4). The solvent was evaporated under reduced pressure, and the residue was purified by column chromatography [EtOAc−petroleum ether (bp 40−60 °C) 2:8].

G. General Method for the Removal of Boc Groups. To a solution of 4 N HCl in MeOH (5.0 mL, 20 mmol), an *N*-Boc-protected component (2 mmol) was added. After being stirred at room temperature for 30 min, the mixture was evaporated, dry Et_2O was added, and the product was filtered and used for coupling with a 2-hydroxy acid.

H. General Method for Catalytic Hydrogenation. To a solution of the substrate (2.00 mmol) in MeOH (10 mL) (through which N_2 had been passed for 5 min), 10% Pd/C catalyst (22 mg, 0.02 mmol) was added. The reaction mixture was stirred under H_2 atmosphere overnight at room temperature. The catalyst was removed by filtration through a pad of Celite, and the organic solvent was evaporated under reduced pressure. The residue was used for the next step.

I. Removal of *tert*-Butyl Groups. To a stirred solution of a *tert*-butyl ester (1 mmol) in CH_2Cl_2 (10 mL), CF_3COOH (10 mL) was added. After the mixture was stirred for 1 h at room temperature, the solvent was evaporated under reduced pressure, and the residue was treated consecutively with petroleum ether (bp 40−60 °C), Et_2O, and dry Et_2O. The residue was purified by column chromatography using EtOAc as eluent

Compounds 10a,b were synthesized from compounds **8a**,**b** by procedure A.

Methyl 4-[(2-hydroxyoctanoyl)amino]butanoate (10a): yield 92%; white solid; mp 68−69 °C; ^{1}H NMR δ 6.75 (1H, t, *J* = 7 Hz, NH), 4.04 (1H, m, CH), 3.67 (3H, s, $COOCH_3$), 3.28 (2H, m, C*H*$_2$NH), 3.19 (1H, br, OH), 2.37 (2H, t, *J* = 7 Hz, C*H*$_2$$COOCH_3$), 1.83 (4H, m, 2 × CH_2), 1.65−1.20 (8H, m, 4 × CH_2), 0.83 (3H, t, *J* = 7 Hz, CH_3); ^{13}C NMR δ 174.1, 173.7, 72.2, 51.7, 38.4, 31.7, 31.0, 29.1, 24.9, 22.6, 14.1.

Methyl 4-[(2-hydroxyhexadecanoyl)amino]butanoate (10b): yield 67%; white solid; mp 90−91 °C; ^{1}H NMR δ 6.79 (1H, t, *J* = 7 Hz, NH), 4.05 (1H, m, CH), 3.67 (3H, s, $COOCH_3$), 3.27 (3H, m, C*H*$_2$NH, OH), 2.39 (2H, t, *J* = 7 Hz, C*H*$_2$-$COOCH_3$), 1.83 (4H, m, C*H*$_2$$CH_2COOCH_3$, C*H*$_2$CH), 1.65−1.20 (24H, m, 12 × CH_2), 0.83 (3H, t, *J* = 7 Hz, CH_3); ^{13}C NMR δ 174.3, 173.8, 72.2, 51.7, 38.4, 31.9, 31.3, 29.6, 24.9, 22.6, 14.1.

AX005 and AX006 were synthesized from compounds **10a**,**b** by procedures B and C.

4-[(2-Oxooctanoyl)amino]butanoic acid (AX005): yield 68%; white solid; mp 79−80 °C; ^{1}H NMR δ 7.22 (1H, m, NH), 3.38 (2H, m, C*H*$_2$NH), 2.91 (2H, t, *J* = 7 Hz, CH_2CO), 2.39 (2H, t, *J* = 7 Hz, C*H*$_2$COOH), 1.88 (2H, m, C*H*$_2$$CH_2COOH$), 1.58 (2H, m, C*H*$_2$$CH_2CO$), 1.25 (6H, m, 3 × CH_2), 0.83 (3H, t, *J* = 7 Hz, CH_3); ^{13}C NMR δ 199.2, 178.4, 160.4, 38.5, 36.7, 31.5, 31.2, 28.6, 24.1, 22.4, 13.9.

4-[(2-Oxohexadecanoyl)amino]butanoic acid (AX006): yield 63%; white solid; mp 113−114 °C; ^{1}H NMR δ 7.20 (1H, m, NH), 3.36 (2H, m, C*H*$_2$NH), 2.90 (2H, t, *J* = 7 Hz, CH_2CO), 2.39 (2H, t, *J* = 7 Hz, C*H*$_2$COOH), 1.87 (2H, m, C*H*$_2$$CH_2$-COOH), 1.56 (2H, m, C*H*$_2$$CH_2CO$), 1.25 (22H, m, 11 × CH_2), 0.83 (3H, t, *J* = 7 Hz, CH_3); ^{13}C NMR δ 199.2, 178.3, 160.4, 38.5, 36.4, 31.9, 30.9, 29.6, 29.3, 29.0, 24.2, 22.6, 14.1.

Methyl 4-[(2-Oxohexadecanoyl)amino]butanoate (AX-010). AX010 was synthesized from **10b** by procedure D. Yield 85%; white solid; mp 70−71 °C; ^{1}H NMR δ 7.18 (1H, m, NH), 3.67 (3H, s, $COOCH_3$), 3.45 (2H, m, C*H*$_2$NH), 2.90 (2H, t, *J* = 7 Hz, CH_2CO), 2.38 (2H, t, *J* = 7 Hz, C*H*$_2$$COOCH_3$), 1.88 (2H, m, C*H*$_2$$CH_2COOCH_3$), 1.58 (2H, m, C*H*$_2$$CH_2CO$), 1.25 (22H, m, 11 × CH_2), 0.83 (3H, t, *J* = 7 Hz, CH_3); ^{13}C NMR δ 199.2, 173.4, 160.3, 51.7, 38.6, 36.5, 31.9, 31.5, 29.6, 29.5, 29.3, 29.0, 24.0, 22.6, 14.1.

4-(Hexadecanoylamino)butyric Acid (AX011). To a stirring solution of 4-aminobutanoic acid (103 mg, 1 mmol) in THF (5 mL) a solution of 1 N NaOH (1.1 mL, 1.1 mmol) was added at 0 °C. Palmitoyl chloride (302 mg, 1.1 mmol) and 1 N NaOH (1.1 mL, 1.1 mmol) were added, and the mixture was stirred for 3 h at room temperature. After acidification with 1 N HCl, the white solid formed was filtered and recrystallized (MeOH). Yield 58%; white solid; mp 104−105 °C; ^{1}H NMR (CD_3OD) δ 7.84 (1H, m, NH), 3.30 (2H, m, C*H*$_2$NH), 2.39 (2H, t, *J* = 7 Hz, C*H*$_2$COOH), 2.18 (2H, t, *J* = 7 Hz, C*H*$_2$CONH) 1.87 (2H, m, C*H*$_2$$CH_2COOH$), 1.56 (2H, m, C*H*$_2$$CH_2CO$), 1.25 (24H, m, 12 × CH_2), 0.88 (3H, t, *J* = 7 Hz, CH_3); ^{13}C NMR δ 177.0, 174.6, 38.9, 35.9, 31.9, 30.9, 29.6, 29.3, 29.0, 24.2, 22.5, 14.1.

Compounds 13, 14a−c, and 15 were synthesized from compounds **11a**−**d** by procedures E and F.

Benzyl (*E*,4*S*)-4-[(*tert*-butoxycarbonyl)amino]oct-2-enoate (13): yield 65%; colorless oil; $[\alpha]_D$ −9.6 (*c* 1, $CHCl_3$); ^{1}H NMR δ 7.36 (5H, m, C_6H_5), 6.90 (1H, dd, J_1 = 15.8 Hz, J_2 = 5.4 Hz, C*H*=CHCOO), 5.96 (1H, dd, J_1 = 15.8 Hz, J_2 = 1.4 Hz, CH=C*H*COO), 5.18 (2H, s, C*H*$_2$$C_6H_5$), 4.51 (1H, d, *J* = 7.8 Hz, NH), 4.28 (1H, m, C*H*NH), 1.62−1.17 [15H, m, 3 × CH_2, $C(CH_3)_3$], 0.90 (3H, t, *J* = 6.0 Hz, CH_3); ^{13}C NMR δ 166.4, 155.4, 149.6, 136.1, 128.7, 128.5, 120.4, 79.9, 66.5, 51.7, 34.6, 28.6, 28.0, 22.6, 14.1; MS (FAB) *m*/*z* (%) 348 (5) [M + H$^+$].

Methyl (*E*,4*S*)-4-[(*tert*-butoxycarbonyl)amino]oct-2-enoate (14a): yield 75%; pale-yellow oil; $[\alpha]_D$ −9.8 (*c* 0.8, CH_3-Cl); ^{1}H NMR δ 6.83 (1H, dd, J_1 = 15.4 Hz, J_2 = 5.2 Hz, C*H*=CHCOO), 5.89 (1H, dd, J_1 = 15.4 Hz, J_2 =1.8 Hz, CH=C*H*COO), 4.64 (1H, d, *J* = 8.0 Hz, NH), 4.24 (1H, m, C*H*NH), 3.70 (3H, s, OCH_3), 1.53 (2H, m, C*H*$_2$CHNH), 1.41 [9H, s, $C(CH_3)_3$], 1.38−1.21 (4H, m, CH_2CH_2), 0.87 (3H, t, *J* = 6.6 Hz, CH_3); ^{13}C NMR δ 166.6, 155.0, 148.9, 119.9, 79.4, 51.4, 34.1, 28.1, 27.6, 22.2, 13.7.

Methyl (*E*,4*S*)-4-[(*tert*-butoxycarbonyl)amino]-5-phenylpent-2-enoate (14b): yield 82%; white solid; mp 73−74 °C; $[\alpha]_D$ +4.5 (*c* 1.0, CH_3Cl); ^{1}H NMR δ 7.20 (m, 5H, C_6H_5), 6.90 (1H, dd, J_1 = 15.4 Hz, J_2 = 4.8 Hz, C*H*=CHCOO), 5.87 (1H, dd, J_1 = 15.4 Hz, J_2 = 1.4 Hz, CH=C*H*COO), 4.49−4.65 (2H, m, NH, CH), 3.70 (3H, m, OCH_3), 2.89 (2H, m, CH_2), 1.40 [s, 9H, $C(CH_3)_3$]; ^{13}C NMR δ 166.5, 154.9, 147.7, 136.3, 129.5, 129.2, 128.7, 128.5, 126.9, 120.6, 79.8, 52.3, 51.6, 40.7, 28.3.

Methyl (*E*,4*S*)-4-[(*tert*-butoxycarbonyl)amino]-6-methylhept-2-enoate (14c): yield 85%; colorless oil; $[\alpha]_D$ −17.0 (*c* 1.0, CH_3Cl); ^{1}H NMR δ 6.80 (1H, dd, J_1 = 15.4 Hz, J_2 = 4.8 Hz, C*H*=CHCOO), 5.87 (1H, dd, J_1 = 15.4 Hz, J_2 = 1.4 Hz, CH=C*H*COO), 4.57 (1H, d, *J* = 8.6 Hz, NH), 4.30 (1H, m, CH), 3.70 (3H, m, OCH_3), 1.67 (2H, m, C*H*$_2$CH), 1.20−1.45 [m, 10H, C*H*$(CH_3)_2$, $C(CH_3)_3$], 0.90 [6H, d, *J* = 6.6 Hz, CH(C*H*$_3$)$_2$]; ^{13}C NMR δ 166.8, 155.0, 149.2, 119.8, 79.6, 51.5, 49.6, 43.6, 28.3, 24.6, 22.6, 22.1.

***tert*-Butyl (*E*,4*S*)-4-[(benzyloxycarbonyl)amino]pent-2-enoate (15):** yield 31%; white solid; $[\alpha]_D$ −11.7 (*c* 1.8, CH_3-Cl); ^{1}H NMR δ 7.34 (5H, m, C_6H_5), 6.76 (1H, dd, J_1 = 15.6 Hz, J_2 = 5.0 Hz, C*H*=CHCOO), 5.73 (1H, dd, J_1 = 15.6 Hz, J_2 = 1.4 Hz, CH=C*H*COO), 5.10 (2H, s, OCH_2), 4.96 (1H, d, *J* = 8.0 Hz, NH), 4.42 (1H, m, C*H*NH), 1.47 [s, 9H, $C(CH_3)_3$], 1.26 (3H, d, *J* = 6.8 Hz, CH_3); ^{13}C NMR δ 165.5, 155.4, 147.5, 136.2, 128.4, 128.1, 122.0, 80.5, 66.8, 47.4, 28.0, 27.9, 20.2.

Benzyl (*E*,4*S*)-4-[(2-Hydroxydodecanoyl)amino]oct-2-enoate (16). Compound **16** was prepared from compound **13** by procedure G (using 4 N HCl in Et_2O instead of 4 N HCl in MeOH) followed by procedure A. Yield 75%; colorless oil; ^{1}H NMR δ 7.35 (5H, m, C_6H_5), 6.90 (1H, dd, J_1 = 15.6 Hz, J_2 = 5.4 Hz, C*H*=CHCOO), 6.65 (1H, m, NH), 5.92 (1H, dd, J_1 = 15.6 Hz, J_2 = 1.8 Hz, CH=C*H*COO), 5.16 (2H, s, C*H*$_2$$C_6H_5$), 4.62 (1H, m, C*H*NH), 4.13 (1H, m, CH), 1.90−1.11 (24H, m, 12 × CH_2), 0.88 (3H, t, *J* = 6.0 Hz, CH_3); ^{13}C NMR δ 173.4,

166.0, 148.4, 135.7, 128.6, 128.3, 120.5, 72.2, 66.4, 49.5, 34.9, 34.7, 33.9, 31.9, 29.5, 29.3, 27.7, 25.0, 24.9, 22.6, 22.4, 22.3, 14.1, 13.8.

Compounds 17a−e were synthesized from compounds **14a−c** by procedures H, G, and A.

Methyl (4*S*)-4-[(2-hydroxypentadecanoyl)amino]octanoate (17a): yield 75%; yellow oil; ^{1}H NMR δ 6.39 (1H, m, NH), 4.08 (2H, m, 2 × CH), 3.66 (3H, s, OCH_3), 2.95 (1H, br, OH), 2.34 (2H, m, CH_2COO), 1.97−1.42 (6H, m, 3 × CH_2), 1.38−1.15 (26H, m, 13 × CH_2), 0.98−0.81 (6H, m, 2 × CH_3); ^{13}C NMR δ 174.2, 173.8, 72.1, 51.7, 48.7, 35.0, 31.9, 30.8, 30.2, 29.6, 29.5, 29.4, 29.3, 27.9, 25.0, 22.6, 22.5, 14.0, 13.9.

Methyl (4*R*)-4-[(2-hydroxyoctanoyl)amino]-5-phenylpentanoate 17b): yield 80%; white solid; mp 75−77 °C; ^{1}H NMR δ 7.23 (5H, m, C_6H_5), 6.31 (1H, d, J = 8.4 Hz, NH), 4.20 (1H, m, CH), 4.01 (1H, m, CH), 3.65 (3H, s, OCH_3), 2.82−2.64 (3H, m, C$H_2C_6H_5$, OH), 2.35 (2H, m, CH_2COO), 2.02−1.15 (12H, m, 6 × CH_2), 0.87 (3H, t, J = 6.6 Hz, CH_3); ^{13}C NMR δ 174.0, 173.6, 137.4, 129.2, 128.6, 126.5, 72.0, 51.7, 49.7, 41.4, 35.0, 31.6, 30.9, 29.0, 24.8, 22.5, 14.0.

Methyl (4*R*)-4-[(2-hydroxypentadecanoyl)amino]-5-phenylpentanoate (17c): yield 86%; white solid; mp 53−55 °C; ^{1}H NMR δ 7.23 (5H, m, C_6H_5), 6.47 (1H, m, NH), 4.20 (1H, m, CH), 4.01 (1H, m, CH), 3.65 (3H, s, OCH_3), 2.90 (1H, br, OH), 2.81 (2H, m, C$H_2C_6H_5$), 2.35 (2H, m, CH_2COO), 2.02−1.42 (4H, m, 2 × CH_2), 1.38−1.12 (22H, m, 11 × CH_2), 0.88 (3H, t, J = 6.6 Hz, CH_3); ^{13}C NMR δ 174.0, 173.8, 137.4, 129.3, 128.4, 126.6, 72.1, 51.7, 49.7, 41.3, 34.9, 31.9, 30.9, 29.6, 29.5, 29.4, 29.3, 29.0, 24.9, 22.6, 14.1.

Methyl (4*R*)-4-[(2-Hydroxydodecanoyl)amino]-6-methylheptanoate (17d): yield 65%; yellow oil; ^{1}H NMR δ 6.35 (1H, m, NH), 4.04 (2H, m, 2 × CH), 3.65 (3H, s, OCH_3), 3.22 (1H, br, OH), 2.34 (2H, t, J = 7.6 Hz, CH_2COO), 1.47−1.95 (5H, m, 2 × CH_2, CH), 1.40−1.18 (18H, m, 9 × CH_2), 0.95−0.78 (9H, m, 3 × CH_3); ^{13}C NMR δ 174.1, 173.8, 72.0, 51.6, 46.8, 46.6, 44.7, 44.5, 34.9, 31.9, 30.8, 30.6, 29.6, 29.5, 29.4, 25.1, 24.9, 23.0, 22.6, 22.0, 14.0.

Methyl (4*R*)-4-[(2-hydroxypentadecanoyl)amino]-6-methylheptanoate (17e): yield 63%; yellow oil; ^{1}H NMR δ 6.37 (1H, m, NH), 4.04 (2H, m, 2 × CH), 3.65 (3H, s, OCH_3), 3.15 (1H, br, OH), 2.34 (2H, t, J = 7.8 Hz, CH_2COO), 1.95−1.47 (5H, m, 2 × CH_2, CH), 1.40−1.18 (24H, m, 12 × CH_2), 0.95−0.81 (9H, m, 3 × CH_3); ^{13}C NMR δ 174.0, 173.8, 72.1, 51.6, 46.8, 46.6, 44.7, 44.5, 34.9, 31.9, 30.8, 30.6, 29.6, 29.5, 29.4, 29.3, 25.0, 24.9, 23.0, 22.6, 22.1, 14.0.

***tert*-Butyl (4*S*)-4-[(2-Hydroxypentadecanoyl)amino]-pentanoate (18).** Compound **18** was synthesized from compound **15** by procedure H (using EtOH instead of MeOH) followed by procedure A (using 2.2 mmol of Et_3N instead of 4.4 mmol of Et_3N). Yield 92%; wax; ^{1}H NMR δ 6.48 (1H, m, NH), 4.12−3.90 (2H, m, 2 × CH), 3.05 (1H, br, OH), 2.27 (2H, t, J = 7.8 Hz, CH_2CO_2), 1.98−1.52 (4H, m, 2 × CH_2), 1.44 [9H, s, C$(CH_3)_3$], 1.40−1.21 (22H, m, 11 × CH_2), 1.16 (3H, d, J = 6.6 Hz, CH_3), 0.88 (3H, t, J = 6.6 Hz, CH_3); ^{13}C NMR δ 173.5, 173.0, 80.6, 72.0, 44.8, 35.0, 32.3, 31.9, 31.6, 29.6, 29.5, 29.4, 29.3, 28.0, 24.9, 22.6, 21.0, 14.1.

(4*S*)-4-[(2-Oxododecanoyl)amino]octanoic Acid (AX007). AX007 was synthesized from compound **16** by procedure H (using EtOH instead MeOH) followed by procedure C. Yield 57%; white solid; mp 50−52 °C; $[\alpha]_D$ −1.8 (*c* 0.5, CH_3Cl); ^{1}H NMR δ 6.86 (1H, d, J = 9.4 Hz, NH), 3.90 (1H, m, C*H*NH), 2.90 (2H, t, J = 7.8 Hz, CH_2COCO), 2.35 (2H, t, J = 6.3 Hz, CH_2COO), 2.01−1.05 (24H, m, 12 × CH_2), 0.87 (3H, t, J = 6.0 Hz, CH_3); ^{13}C NMR δ 199.4, 178.5, 160.1, 49.3, 36.8, 34.7, 31.8, 30.7, 29.8, 29.5, 29.3, 29.2, 29.0, 27.9, 23.1, 22.6, 22.4, 14.1, 13.9; MS (FAB) *m*/*z* (%) 378 (35) [M^+ + Na], 356 (45) [M^+ + H].

AX008 was synthesized in a similar way to AX007. The spectroscopic data were identical to those obtained for the (*S*)-enantiomer. $[\alpha]_D$ −1.6 (*c* 0.5, CH_3Cl).

(4*S*)-4-[(2-Oxopentadecanoyl)amino]pentanoic Acid (AX014). AX014 was synthesized from compound **18** by procedure D followed by procedure I. Yield 63%; white solid; mp 112−113 °C; $[\alpha]_D$ −0.7 (*c* 0.7, CH_3Cl); ^{1}H NMR δ 6.93 (1H, d, J = 9 Hz, NH), 4.03 (1H, m, CH), 2.91 (2H, t, J = 7.8 Hz, CH_2COCO), 2.39 (2H, m, CH_2COOH), 1.85 (2H, m, CH_2), 1.59 (2H, m, CH_2), 1.25 (23H, m, 10 × CH_2, CH_3), 0.88 (3H, t, J = 6.7 Hz, CH_3); ^{13}C NMR δ 199.4, 178.3, 159.7, 45.1, 36.7, 31.9, 31.3, 30.7, 29.6, 29.4, 29.3, 29.0, 23.1, 22.7, 20.7,14.1; MS (FAB) *m*/*z* (%) 356 (100) [M^+ + H].

AX012, AX013, AX016, AX017, and AX024 were synthesized from compounds **17a−e** by procedures B and C.

(4*R*)-6-Methyl-4-[(2-oxopentadecanoyl)amino]heptanoic acid (AX012): yield 67%; white solid; mp 58−59 °C; $[\alpha]_D$ −9.0 (*c* 1.28 CH_3Cl); ^{1}H NMR δ 6.95 (1H, d, J = 9.2 Hz, NH), 4.00 (1H, m, CH), 2.91 (2H, t, J = 7.8 Hz, CH_2COCO), 2.30 (2H, m, CH_2COOH), 1.91 [3H, m, CH_2, C$H(CH_3)_2$], 1.59 (4H, m, 2 × CH_2), 1.25 (20H, m, 10 × CH_2), 0.91 (9H, t, J = 6.8 Hz, 3 × CH_3); ^{13}C NMR δ 199.3, 178.8, 160.0, 47.3, 44.1, 36.8, 31.8, 31.1, 30.4, 29.5, 29.3, 29.2, 28.9, 24.7, 23.0, 22.9, 22.5, 21.9, 13.9; MS (FAB) *m*/*z* (%) 398 (100) [M^+ + H].

(4*R*)-6-Methyl-4-[(2-oxododecanoyl)amino]heptanoic acid (AX013): yield 83%; white solid; mp 55−56 °C; $[\alpha]_D$ −9.5 (*c* 1.3 CH_3Cl); ^{1}H NMR δ 6.83 (1H, d, J = 9 Hz, NH), 4.02 (1H, m, CH), 2.91 (2H, t, J = 7.8 Hz, CH_2COCO), 2.36 (2H, m, CH_2COOH), 1.91 [3H, m, CH_2, C$H(CH_3)_2$], 1.59 (4H, m, 2 × CH_2), 1.25 (14H, m, 7 × CH_2), 0.91 (9H, t, J = 6.8 Hz, 3 × CH_3); ^{13}C NMR δ 199.4, 178.4, 160.1, 47.4, 44.3, 36.8, 31.8, 30.7, 30.4, 29.5, 29.4, 29.3, 29.2, 29.0, 24.9, 23.2, 22.9, 22.6, 22.0, 14.1; MS (FAB) *m*/*z* (%) 400 (100) [M^+ + 2Na − 1], 378 (46) [M^+ + Na].

(4*R*)-4-[(2-Oxopentadecanoyl)amino]-5-phenylpentanoic acid (AX016): yield 82%; white solid; mp 91−92 °C; $[\alpha]_D$ −6.1 (*c* 0.8, CH_3Cl); ^{1}H NMR δ 7.33−7.14 (5H, m, C_6H_5), 6.97 (1H, d, J = 9 Hz, NH), 4.20 (1H, m, CH), 2.86 (4H, m, C$H_2C_6H_5$, CH_2COCO), 2.37 (2H, t, J = 7.2 Hz, CH_2COOH), 1.95 (1H, m, C*H*H), 1.81 (1H, m, C*H*H), 1.56 (2H, m, CH_2), 1.26 (20H, m, 10 × CH_2), 0.89 (3H, t, J = 6.8 Hz, CH_3); ^{13}C NMR δ 199.2, 178.0, 160.0, 136.9, 129.2, 128.6, 126.8, 50.3, 41.1, 36.7, 31.9, 30.7, 29.6, 29.4, 29.3, 29.0, 28.9, 23.1, 22.7, 14.1; MS (FAB) *m*/*z* (%) 476 (65) [M^+ + 2Na − 1], 454 (30) [M^+ + Na].

(4*R*)-4-[(2-Oxooctanoyl)amino]-5-phenylpentanoic acid (AX017): yield 75%; pale-yellow solid; mp 94−96 °C; $[\alpha]_D$ −8.7 (*c* 0.24, CH_3Cl); ^{1}H NMR δ 7.38−7.14 (5H, m, C_6H_5), 6.95 (1H, d, J = 9 Hz, NH), 4.20 (1H, m, CH), 2.86 (4H, m, C$H_2C_6H_5$, CH_2COCO), 2.37 (2H, t, J = 7.4 Hz, CH_2COO), 1.97 (1H, m, C*H*H), 1.78 (1H, m, C*H*H), 1.57 (2H, m, CH_2), 1.26 (6H, m, 3 × CH_2), 0.87 (3H, t, J = 6.8 Hz, CH_3); ^{13}C NMR δ 199.2, 177.6, 160.0, 136.9, 128.7, 128.1, 127.0, 50.3, 41.1, 36.7, 31.5, 28.7, 26.3, 22.4, 14.0; MS (FAB) *m*/*z* (%) 334 (57) [M^+ + H].

(4*S*)-4-[(2-Oxopentanodecanoyl)amino]octanoic acid (AX024): yield 56%; white solid; mp 74−75 °C; $[\alpha]_D$ −1.6 (*c* 0.6, CH_3Cl); ^{1}H NMR δ 6.85 (1H, d, J = 9.8 Hz, NH), 3.93 (1H, m, CH), 2.92 (2H, t, J = 7.4 Hz, CH_2COCO), 2.37 (2H, t, J = 7.1 Hz, CH_2COO), 2.03−1.44 (6H, m, 3 × CH_2), 1.42−1.17 (24H, m, 12 × CH_2), 0.91−0.83 (6H, t, J = 6.3 Hz, 2 × CH_3); ^{13}C NMR δ 199.4, 178.1, 160.1, 49.3, 36.8, 34.7, 31.9, 30.8, 30.0, 29.6, 29.4, 29.3, 29.0, 28.0, 23.2, 22.7, 22.4, 14.1, 13.9; MS (FAB) *m*/*z* (%) 398 (100) [M^+ + H].

AX018 and AX019 were synthesized from Boc-L-prolinol by procedures E−H and A−C.

(*S*)-3-[1-(2-Oxopentadecanoyl)pyrrolidin-2-yl]propionic acid (AX018): Pale-yellow solid; mp 55−56 °C; $[\alpha]_D$ −42.7 (*c* 1.0, CH_3Cl); ^{1}H NMR δ 4.21 (1H, m, CH), 3.54 (2H, m, CH_2-NH), 2.84 (2H, m, CH_2COCO), 2.35 (2H, m, CH_2COOH), 2.01−1.41 (8H, m, 4 × CH_2), 1.25 (20H, m, 10 × CH_2), 0.86 (3H, t, J = 6.6 Hz, CH_3); ^{13}C NMR δ 201.0, 177.9, 163.6, 57.1, 47.2, 39.2, 31.8, 31.1, 29.6, 29.4, 29.3, 29.1, 29.0, 28.4, 24.2, 22.9, 22.6, 14.1; MS (FAB) *m*/*z* (%) 426 (100) [M^+ + 2Na − 1], 404 (27) [M^+ + Na].

(*S*)-3-[1-(2-Oxododecanoyl)pyrrolidin-2-yl]propionic acid (AX019): yellow oil; $[\alpha]_D$ −37.7 (*c* 0.5, CH_3Cl); ^{1}H NMR δ 4.21 (1H, m, CH), 3.54 (2H, m, CH_2NH), 2.84 (2H, m, CH_2-COCO), 2.35 (2H, m, CH_2COOH), 2.01−1.41 (8H, m, 4 × CH_2), 1.25 (14H, m, 7 × CH_2), 0.86 (3H, t, J = 7 Hz, CH_3); ^{13}C NMR δ 201.0, 177.6, 164.1, 57.1, 47.2, 39.3, 31.9, 31.2, 29.5, 29.4, 29.3, 29.1, 28.6, 24.2, 22.9, 22.6, 14.1.

(2*S*)-2-[(*tert*-Butoxycarbonyl)amino]-3-decyloxypropanoic Acid (20). To a solution of **19** (20 g, 100 mmol) in DMF (500 mL), NaH (8.2 g, 220 mmol) was added at 0 °C. Iododecane (24 mL, 110 mmol) was added when the H_2 release had finished. The reaction mixture was stirred for 5 h at room temperature. The organic solvent was evaporated under reduced pressure, and the product was extracted with brine. The product was purified by column chromatography ($CHCl_3$, $CHCl_3$−MeOH, 9:1). Yield 68%; oil; $[\alpha]_D$ +5.5 (*c* 1.75, $CHCl_3$); ^{1}H NMR δ 4.88 (H, m, OCONH), 4.23 (1H, m, CHNH), 3.69 (2H, d, CHCH_2O, J = 13.6 Hz), 3.43 (2H, t, OCH_2 J = 4.8), 1.52−1.21 (25H, m, 8 × $CH_2C(CH_3)_3$), 0.88 (3H, t, J = 6.6 Hz, CH_3); ^{13}C NMR δ 176.1, 154.7, 79.0, 71.3, 54.0, 36.4, 31.2, 28.9, 28.6, 27.7, 27.6, 24.4, 22.4, 22.0, 14.0.

(2*S*)-2-[(*tert*-Butoxycarbonyl)amino]-3-(decyloxy)propanol (21). To a stirred solution of **20** (0.34 g, 1.0 mmol) in dry THF (5 mL) at −10 °C, NMM (0.11 mL, 1.0 mmol) was added, followed by ClCOOEt (0.096 mL, 1.0 mmol). After 10 min, $NaBH_4$ (0.11 g, 3.0 mmol) was added in one portion. MeOH (10 mL) was then added dropwise to the mixture over a period of 10 min at 0 °C. The solution was stirred for an additional 10 min and then neutralized with 1 M $KHSO_4$. The organic solvents were evaporated under reduced pressure, and the product was extracted with EtOAc (3 × 7 mL). The organic phase was washed with 1 M $KHSO_4$ (5 mL), H_2O (10 mL), 5% $NaHCO_3$ (5 mL), and H_2O (10 mL) and dried, and the solvent was evaporated under reduced pressure. The residue was purified by crystallization with Et_2O/petroleum ether. Yield 75%; oil; $[\alpha]_D$ +6.4 (*c* 1, $CHCl_3$); ^{1}H NMR δ 4.21 (1H, m, OCONH), 3.82−3.25 (7H, m, 2 × CH_2O, CH_2OH, CHNH), 1.69−1.08 (25H, m, 8 × CH_2, $C(CH_3)_3$), 0.88 (3H, t, J = 6.6 Hz, CH_3); ^{13}C NMR δ 156.2, 79.6, 71.8, 64.4, 51.4, 31.8, 29.4, 29.3, 28.4, 28.3, 26.0, 22.6, 14.1; MS (FAB): *m*/*z* (%) 332 (45) [M^+ + H].

Benzyl (*E*,4*R*)-4-[(*tert*-Butoxycarbonyl)amino]-5-decyloxypent-2-enoate (22). Compound **22** was synthesized from compound **21** by procedures E and F. Yield 52%; oil; $[\alpha]_D$ −2.3 (*c* 1, AcOEt); ^{1}H NMR δ 7.37 (5H, C_6H_5), 6,97 (1H, dd, J = 14.6 Hz, J = 4.2 Hz, C*H*═CHCOOBn), 6.03 (1H, dd, J = 14.6 Hz, J = 1.4 Hz, CH═C*H*COOBn), 4.18 (s, 2H, C$H_2C_6H_5$), 4.00 (1H, d, J = 7.0 Hz, OCONH), 4.45 (1H, m, C*H*NH), 3.51 (2H, d, CHCH_2O, J = 4.4 Hz), 3.41 (2H, t, CH_2CH_2O, J = 6,4 Hz), 1.62−1.16 (25H, m, 8'CH_2., $C(CH_3)_3$), 0.9 (3H, t, J = 6.0 Hz, CH_3); ^{13}C NMR δ 166.0, 154.8, 146.9, 139.8, 128.3, 126.8, 121.4, 79.6, 71.6, 66.2, 51.4, 31.8, 29.4, 29.2, 28.4, 28.3, 26.0, 22.6, 14.1.

Benzyl (*E*,4*R*)-5-(Decyloxy)-4-[(2-hydroxydodecanoyl)amino]pent-2-enoate (23). Compound **23** was prepared from **22** by procedure G (using 4 N HCl in Et_2O instead of 4 N HCl in MeOH) followed by procedure A. Yield 65%; oil; ^{1}H NMR δ 7.36 (5H, C_6H_5),7.02−6.90 (2H, m, CONH, C*H*═CHCOOBn), 6.00 (1H, d, J = 14.6 Hz, CH═C*H*COOBn), 4.17 (2H, s, C$H_2C_6H_5$), 4.81 (1H, m, C*H*NH), 4.14 (1H, m, C*H*OH), 3.54 (2H, d, CHCH_2O, J = 4.0 Hz), 3.40 (2H, t, CH_2CH_2O, J = 6,2 Hz), 1.81−1.08 (34H, m, 17 × CH_2), 0.88 (3H, t, J = 6.0 Hz, CH_3); ^{13}C NMR δ 173.4, 157.6, 146.0, 134.7, 128.4, 128.3, 121.9, 72.2, 71.7, 71.4, 66.6, 49.6, 31.8, 29.6, 29.4, 29.2, 24.9, 22.6, 14.1; MS (FAB) *m*/*z* (%) 560 (20) [M^+ + H].

(4*R*)-5-(Decyloxy)-4-[(2-oxododecanoyl)amino]pentanoic Acid (AX027). AX027 was synthesized from compound **23** by procedure H (using EtOH instead MeOH) followed by procedure C. Yield 46%; white solid; mp 54−56 °C; $[\alpha]_D$ −16.2 (*c* 0.5, $CHCl_3$); ^{1}H NMR δ 7.24 (1H, d, CONH, J = 9.,4 Hz), 4.08 (1H, m, C*H*NH), 3.42 (4H, m, 2'CH_2O), 2.90 (2H, t, CH_2-COCO, J = 7.8 Hz), 2.39 (2H, t, CH_2COOH, J = 7.0 Hz), 1.99 (2H, m, CH_2CH_2COOH), 1.58 (4H, m, CH_2CH_2COCO, CH_2-CH_2O), 1.26 (28H, m, 14 × CH_2), 0.88 (3H, t, J = 6,0 Hz, CH_3); ^{13}C NMR δ 199.0, 178.4, 160.0, 71.6, 69.7, 48.7, 36.8, 31.8, 29.6, 29.4, 29.3, 29.0, 26.0, 23.1, 22.6, 14.1; MS (FAB) *m*/*z* (%) 470 (15) [M^+ + H].

AX026 was synthesized starting from D-serine in a similar way as AX027. The spectroscopic data were identical to those obtained for the (*S*)-enantiomer. $[\alpha]_D$ +15.6 (*c* 0.5, CH_3Cl).

J. Phospholipase A_2 Activity Assays. Phospholipase A_2 assays were performed as described in the Supporting Information of our initial study.[17] Unless otherwise indicated, the phospholipid/detergent mixed micelles were composed of substrate, PAPC, PIP_2, and the detergent TX-100 at a ratio of 97:3:400 (μM), respectively. Where indicated, the mole fraction of each of the 2-oxoamide compounds was varied within otherwise identical phospholipid substrate micelles and was plotted against the percent inhibition of GIVA PLA_2 activity relative to a control with dimethyl sulfoxide (DMSO) present.

K. Cell Maintenance. $P388D_1$ murine macrophages were maintained at 37 °C in a humidified 10% CO_2 atmosphere. Cells were grown in Iscove's modified Dulbecco's medium (IMDM) with 10% fetal calf serum (HyClone Labs, Provo, UT), 100 units/mL penicillin, and 100 mg/mL streptomycin (Invitrogen, Carlsbad, CA). Cells were routinely passaged every 2−3 days when the cells reached 75−80% confluence. Cells used for stimulation were plated at a density of 106 cells per well in standard 12-well tissue culture plates and were allowed to adhere overnight.

L. Cell Stimulation. The standard regimen for treatment of the $P338D_1$ cells with either LPS alone or LPS combined with PAF has been previously described.[37−40] Briefly cells were washed twice with starvation media (IMDM containing 0.2% fetal calf serum, 100 units/mL penicillin, and 100 mg/mL streptomycin) and then incubated with 1 mL of starvation media for 1 h. Cells were then exposed to either 100 ng/mL LPS for 18 h or 200 ng/mL LPS for 1 h. Cells treated with 200 ng/mL LPS were then exposed to 100 nM PAF for 15 min. At the end of the stimulation, the medium was removed from the cells, cleared of cellular debris by centrifugation, and analyzed for PGE_2 levels by enzyme-linked immunoassay (Cayman Chemical, Ann Arbor, MI). Inhibitor compounds were dissolved in DMSO and diluted into starvation media prior to addition to cells; the DMSO concentration was kept below 0.5% v/v in all studies. All inhibitors were added 30 min prior to stimulation.

M. Inhibition of Carrageenan-Induced Edema. Edema was induced in the right hind paw of Fisher 344 rats (150−200 g) by the intradermal injection of 0.1 mL of 2% w/v carrageenan (K100, commercially available) in sterilized saline into the right footpad.[41] Both sexes were used. Pregnant females were excluded. The animals, bred in our laboratory, were housed under standard conditions and received a diet of commercial food pellets and water ad libitum. During the experiment period, food, but not water, was withheld. These studies were in accordance with recognized guidelines on animal experimentation (Guidelines for the Care and Use of Laboratory Animals, published by the Greek Goverment 160/1991, based on EU regulations 86/609).

The tested compounds were suspended in a few drops of Tween 80, ground in a mortar before use, diluted dropwise with water to the appropriate concentrations, and were given intraperitoneally (ip) at the same time as the carrageenin. The rats were euthanized 3.5 h after carrageenin injection. The experiment was repeated twice for each compound (two groups of five animals) at four different concentrations. The difference between the weight of the injected and uninjected paws was calculated for each animal. The change in paw weight was compared with that in control animals (injected with water) and expressed as a percent inhibition of the edema (CPE % values). Indomethacin in 0.01 mmol/kg (47%) was administered as a standard comparative drug. Values for CPE % are the mean from two different experiments with a standard error of the mean less than 10%. Statistical studies were made with Student's *t*-test.

N. Analgesic Screen. The writhing reflex was used. To a group of seven Fischer rats (150−200 g) compounds tested for anti-inflammatory activity were administered ip with a dose equivalent to the ED_{50} 30 min prior to the ip administration of 1 mL/100 g of body weight of 0.6% acetic acid.[42] Sodium acetylsalicylate was administered ip as a standard drug. After 5 min, the number of stretches characterized by repeated contractions of the abdominal musculature accompanied by

extension of the hind limbs was counted every 5 min for the next 30 min. The total number of writhes exhibited by each animal in the test group was recorded and compared to that of a vehicle-treated control group. The percent analgesic activity (aa) was calculated as

$$\% \text{ aa} = \frac{n - n'}{n} \times 100$$

where n is the average number of writhes of the control group and n' is the average number of writhes of the test group.

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Supporting Information Available: Elemental analysis results for the compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

Appendix

Abbreviations. AA, arachidonic acid; AATFK, arachidonoyl trifluoromethyl ketone; AcNH-TEMPO, 4-acetamido-2,2,6,6-tetramethylpiperidine-1-yloxy free radical; Bn, benzyl; Boc, *tert*-butoxycarbonyl; DMF, *N*,*N*′-dimethylformamide; DMSO, dimethyl sulfoxide; EtOAc, ethyl acetate; GABA, γ-aminobutyric acid; GIVA PLA_2, human Group IVA cytosolic phospholipase A_2; HOBt, 1-hydroxybenzotriazole; IMDM, Iscove's modified Dulbecco's medium; ip, intraperitoneally; LPS, lipopolysaccharide; MAFP, methylarachidonoyl fluorophosphonate; NMM, *N*-methylmorpholine; PAF, platelet activating factor; PAPC, 1-palmitoyl-2-arachidonoylphosphatidylcholine; PDC, pyridinium dichromate; PGE_2, prostaglandin E_2; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLA_2, phospholipase A_2; THF, tetrahydrofuran; TX-100, Triton X-100; WSCI, 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide; Z, benzyloxycarbonyl.

References

(1) Six, D. A.; Dennis, E. A. The expanding superfamily of phospholipase A_2 enzymes: classification and characterization. *Biochim. Biophys. Acta* **2000**, *1488*, 1−19.
(2) Pickard, R. T.; Chiou, X. G.; Strifler, B. A.; DeFelippis, M. R.; Hyslop, P. A.; Tebbe, A. L.; Yee, Y. K.; Reynolds, L. J.; Dennis, E. A.; Kramer, R. M.; Sharp, J. D. Identification of essential residues for the catalytic function of 85-kDa cytosolic phospholipase A_2. Probing the role of histidine, aspartic acid, cysteine, and arginine. *J. Biol. Chem.* **1996**, *271*, 19225−19231.
(3) Dessen, A.; Tang, J.; Schmidt, H.; Stahl, M.; Clark, J. D.; Seehra, J.; Somers, W. S. Crystal structure of human cytosolic phospholipase A_2 reveals a novel topology and catalytic mechanism. *Cell* **1999**, *97*, 349−360.
(4) Larsson, P. K.; Claesson, H. E.; Kennedy, B. P. Multiple splice variants of the human calcium-independent phospholipase A_2 and their effect on enzyme activity. *J. Biol. Chem.* **1998**, *273*, 207−214.
(5) Ackermann, E. J.; Conde-Frieboes, K.; Dennis, E. A. Inhibition of macrophage Ca^{2+}-independent phospholipase A_2 by bromoenol lactone and trifluoromethyl ketones. *J. Biol. Chem.* **1995**, *270*, 445−450.
(6) Conde-Frieboes, K.; Reynolds, L. J.; Lio, Y.; Hale, M.; Wasserman, H. H.; Dennis, E. A. Activated ketones as inhibitors of intracellular Ca^{2+}-dependent and Ca^{2+}-independent phospholipase A_2. *J. Am. Chem. Soc.* **1996**, *118*, 5519−5525.
(7) Lio, Y.; Reynolds, L. J.; Balsinde, J.; Dennis, E. A. Irreversible inhibition of Ca^{2+}-independent phospholipase A_2 by methyl arachidonyl fluorophosphonate. *Biochim. Biophys. Acta* **1996**, *1302*, 55−60.
(8) Ghomashchi, F.; Loo, R.; Balsinde, J.; Bartoli, F.; Apitz-Castro, R.; Clark, J. D.; Dennis, E. A.; Gelb, M. H. Trifluoromethyl ketones and methyl fluorophosphonates as inhibitors of group IV and VI phospholipases A_2: structure−function studies with vesicle, micelle, and membrane assays. *Biochim. Biophys. Acta* **1999**, *1420*, 45−56.
(9) Bonventre, J. V.; Huang, Z.; Taheri, M. R.; O'Leary, E.; Li, E.; Moskowitz, M. A.; Sapirstein, A. Reduced fertility and postischaemic brain injury in mice deficient in cytosolic phospholipase A_2. *Nature* **1997**, *390*, 622−625.
(10) Klivenyi, P.; Beal, M. F.; Ferrante, R. J.; Andreassen, O. A.; Wermer, M.; Chin, M. R.; Bonventre, J. V. Mice deficient in group IV cytosolic phospholipase A_2 are resistant to MPTP neurotoxicity. *J. Neurochem.* **1998**, *71*, 2634−2637.
(11) Uozumi, N.; Kume, K.; Nagase, T.; Nakatani, N.; Ishii, S.; Tashiro, F.; Komagata, Y.; Maki, K.; Ikuta, K.; Ouchi, Y.; Miyazaki, J.; Shimizu, T. Role of cytosolic phospholipase A_2 in allergic response and parturition. *Nature* **1997**, *390*, 618−622.
(12) Fujishima, H.; Sanchez Mejia, R. O.; Bingham, C. O., III; Lam, B. K.; Sapirstein, A.; Bonventre, J. V.; Austen, K. F.; Arm, J. P. Cytosolic phospholipase A_2 is essential for both the immediate and the delayed phases of eicosanoid generation in mouse bone marrow-derived mast cells. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 4803−4807.
(13) Uozumi, N.; Shimizu, T. Roles for cytosolic phospholipase $A_2\alpha$ as revealed by gene-targeted mice. *Prostaglandins Other Lipid Mediators* **2002**, *68*−*69*, 59−69.
(14) Nagase, T.; Uozumi, N.; Ishii, S.; Kume, K.; Izumi, T.; Ouchi, Y.; Shimizu, T. Acute lung injury by sepsis and acid aspiration: a key role for cytosolic phospholipase A_2. *Nat. Immunol.* **2000**, *1*, 42−46.
(15) Song, H.; Lim, H.; Paria, B. C.; Matsumoto, H.; Swift, L. L.; Morrow, J.; Bonventre, J. V.; Dey, S. K. Cytosolic phospholipase $A_2\alpha$ is crucial for "on-time" embryo implantation that directs subsequent development. *Development* **2002**, *129*, 2879−2889.
(16) Serhan, C. N. Preventing injury from within, using selective $cPLA_2$ inhibitors. *Nat. Immunol.* **2000**, *1*, 13−15.
(17) Kokotos, G.; Kotsovolou, S.; Six, D. A.; Constantinou-Kokotou, V.; Beltzner, C. C.; Dennis, E. A. Novel 2-oxoamide inhibitors of Group IVA phospholipase A_2. *J. Med. Chem.* **2002**, *45*, 2891−2893.
(18) Street, I. P.; Lin, H.-K.; Laliberte, F.; Ghomaschi, F.; Wang, Z.; Perrier, H.; Tremblay, N. M.; Huang, Z.; Weeck, P. K.; Gelb, M. H. Slow- and tight-binding inhibitors of the 85-kDa human phospholipase A_2. *Biochemistry* **1993**, *32*, 5935−5940.
(19) Trimble, L. A.; Street, I. P.; Perrier, H.; Tremblay, N. M.; Weech, P. K.; Berstein, M. A. NMR structural studies of the tight complex between a trifluoromethyl ketone inhibitor and the 85-kDa human phospholipase A_2. *Biochemistry* **1993**, *32*, 12560−12565.
(20) Huang, Z.; Payette, P.; Abdullah, K.; Cromlish, W. A.; Kennedy, B. P. Functional identification of the active-site nucleophile of the human 85-kDa cytosolic phospholipase A_2. *Biochemistry* **1996**, *35*, 3712−3721.
(21) Conde-Frieboes, K.; Reynolds, L. J.; Lio, Y.-C.; Hale, M. R.; Wasserman, H. H.; Dennis, E. A. Activated ketones as inhibitors of intracellular Ca^{2+}-dependent and Ca^{2+}-independent phospholipase A_2. *J. Am. Chem. Soc.* **1996**, *118*, 5519−5525.
(22) Removed in proofs.
(23) Lehr, M. Structure−activity relationships of (4-acylpyrrol-2-yl)-alkanoic acids as inhibitors of the cytosolic phospholipase A_2: variation of the substitutents in positions 1, 3, and 5. *J. Med. Chem.* **1997**, *40*, 3381−3392.
(24) Lehr, M.; Klimt, M.; Elfringhoff, A. S. Novel 3-dodecanoylindone-2-carboxylic acid inhibitors of cytosolic phospholipase A_2. *Bioorg. Med. Chem. Lett.* **2001**, *11*, 2569−2572.
(25) Seno, K.; Okuno, T.; Nishi, K.; Murakami, Y.; Watanabe, F.; Matsuura, T.; Wada, M.; Fujii, Y.; Yamada, M.; Ogawa, T.; Okada, T.; Hashizume, H.; Kii, M.; Hara, S.-I.; Hagishita, S.; Nakamoto, S.; Yamada, K.; Chikazawa, Y.; Ueno, M.; Teshirogi, I.; Ono, T.; Ohtani, M. Pyrrolidine inhibitors of human cytosolic phospholipase A_2. *J. Med. Chem.* **2000**, *43*, 1041−1044.
(26) Seno, K.; Okuno, T.; Nichi, K.; Murakami, Y.; Yamada, K.; Nakamoto, K.; Ono, T. Pyrrolidine inhibitors of human cytosolic phospholipase A_2. Part 2: Synthesis of potent and crystallized 4-triphenylmethylthio derivative "pyrrophenone". *Bioorg. Med. Chem. Lett.* **2001**, *11*, 587−590.
(27) (a) Chiou, A.; Markidis, T.; Constantinou-Kokotou, V.; Verger, R.; Kokotos, G. Synthesis and study of a lipolytic α-keto amide inhibitor of pancreatic lipase. *Org. Lett.*, **2000**, *2*, 347−350. (b) Chiou, A.; Verger, R.; Kokotos, G. Synthetic routes and lipase-inhibiting activity of long-chain α-keto amides. *Lipids* **2001**, *36*, 535−542.

(28) (a) Kokotos, G.; Verger, R.; Chiou, A. Synthesis of 2-oxo amide triacylglycerol analogues and study of their inhibition effect on pancreatic and gastric lipases. *Chem.—Eur. J.* **2000**, *6*, 4211−4217. (b) Kotsovolou, S.; Chiou, A.; Verger, R.; Kokotos, G. Bis-2-oxo amide triacylglycerol analogues: a novel class of potent human gastric lipase inhibitors. *J. Org. Chem.* **2001**, *66*, 962−967.

(29) Connolly, S.; Bennion, C.; Botterell, S.; Croshaw, P. J.; Hallam, C.; Hardy, K.; Hartopp, P.; Jackson, C. G.; King, S. J.; Lawrence, L.; Mete, A.; Murray, D.; Robinson, D. H.; Smith, G. M.; Stein, L.; Walters, I.; Wells, E.; Withnall, W. J. Design and synthesis of a novel and potent series of inhibitors of cytosolic phospholipase A_2 based on a 1,3-disubstituted propan-2-one skeleton. *J. Med. Chem.* **2002**, *45*, 1348−1362.

(30) Sheehan, J. C.; Cruickshank, P. A.; Boshart, G. L. A convenient synthesis of water-soluble carbodiimides. *J. Org. Chem.* **1961**, *26*, 2525−2528.

(31) (a) Leanna, M. R.; Sowin, T. J.; Morton, H. E. Synthesis of α-amino and α-alkoxy aldehydes via oxammonium oxidation. *Tetrahedron Lett.* **1992**, *33*, 5029−5032. (b) Ma, Z.; Bobbitt, J. M. Organic oxoammonium salts: a new convenient method for the oxidation of alcohols to aldehydes and amines. *J. Org. Chem.* **1991**, *56*, 6110−6114.

(32) Kokotos, G. A convenient one-pot conversion of N-protected amino acids and peptides into alcohols. *Synthesis* **1990**, 299−301.

(33) Kokotos, G.; Noula, C. Selective one-pot conversion of carboxylic acids into alcohols. *J. Org. Chem.* **1996**, *61*, 6994−6996.

(34) Mosior, M.; Six, D. A.; Dennis, E. A. Group IV cytosolic phospholipase A_2 binds with high affinity and specificity to phosphatidylinositol 4,5-bisphosphate resulting in dramatic increases in activity. *J. Biol. Chem.* **1998**, *273*, 2184−2191.

(35) Six, D. A.; Dennis, E. A. Essential Ca^{2+}-independent role of the Group IVA cytosolic phospholipase A_2 C2 domain for interfacial activity. *J. Biol. Chem.* **2003**, *278*, 23842−23850.

(36) Beaton, H. G.; Bennion, C.; Connolly, S.; Cook, A. R.; Gensmantel, N. P.; Hallam, C.; Hardy, K.; Hitchin, B.; Jackson, C. J.; Robinson, D. H. The discovery of a new nonphospholipid inhibitors of the secretory phospholipases A_2. *J. Med. Chem.* **1994**, *37*, 557−559.

(37) Balsinde, J.; Barbour, S. E.; Bianco, I. D.; Dennis, E. A. Arachidonic acid mobilization in $P388D_1$ macrophages is controlled by two distinct Ca^{2+}-dependent phospholipase A_2 enzymes. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 11060−11064.

(38) Shinohara, H.; Balboa, M. A.; Johnson, C. A.; Balsinde, J.; Dennis, E. A. Regulation of delayed prostaglandin production in activated $P388D_1$ macrophages by group IV cytosolic and group V secretory phospholipase A_2s. *J. Biol. Chem.* **1999**, *274*, 12263−12268.

(39) Balsinde, J.; Shinohara, H.; Lefkowitz, L. J.; Johnson, C. A.; Balboa, M. A.; Dennis, E. A. Group V phospholipase A_2 dependent induction of cyclooxygenase-2 in macrophages. *J. Biol. Chem.* **1999**, *274*, 25967−25970.

(40) Balsinde, J.; Dennis, E. A. Distinct roles in signal transduction for each of the phospholipase A_2 enzymes present in $P388D_1$ macrophages. *J. Biol. Chem.* **1996**, *271*, 6758−6765.

(41) Kontogiorgis, C.; Hadjipavlou-Litina, D. Biological evaluation of several coumarin derivatives designed as possible anti-inflammatory/antioxidant agents. *J. Enzyme Inhib. Med. Chem.* **2003**, *18*, 63−69.

(42) Hadjipavlou-Litina, D.; Rekka, E.; Hadjipetrou-Kourounakis, L.; Kourounakis, P. N. Synthesis and biological studies of some novel anti-inflammatory aryl-hydroxy-amino-ketones. *Eur. J. Med. Chem.* **1991**, *26*, 85−90.

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