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2-Oxoamide inhibitors of phospholipase A_2 activity and cellular arachidonate release based on dipeptides and pseudodipeptides

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ABSTRACT

A series of 2-oxoamides based on dipeptides and pseudodipeptides were synthesized and their activities towards two human intracellular phospholipases A_2 (GIVA cPLA$_2$ and GVIA iPLA$_2$) and one human secretory phospholipase A_2 (GV sPLA$_2$) were evaluated. Derivatives containing a free carboxyl group are selective GIVA cPLA$_2$ inhibitors. A derivative based on the ethyl ester of an ether pseudodipeptide is the first 2-oxoamide, which preferentially inhibits GVIA iPLA$_2$. The effect of 2-oxoamides on the generation of arachidonic acid from RAW 264.7 macrophages was also studied and it was found that selective GIVA cPLA$_2$ inhibitors preferentially inhibited cellular arachidonic acid release; one pseudodipeptide gave an IC_{50} value of 2 μM.

1. Introduction

Phospholipase A_2 (PLA$_2$) enzymes catalyze the hydrolysis of the *sn*-2 ester bond of glycerophospholipids producing free fatty acids and lysophospholipids.[1,2] The PLA$_2$ superfamily currently consists of 15 groups and many subgroups of which a number of enzymes differ in primary sequence, structure and catalytic mechanism.[1] However, the three predominant types of phospholipase A_2 (PLA$_2$) found in human tissues are the cytosolic (such as the GIVA cPLA$_2$), the secreted (such as the GV sPLA$_2$) and the calcium-independent (such as the GVIA iPLA$_2$) enzymes. GIVA cPLA$_2$ preferentially hydrolyzes membrane phospholipids containing arachidonic acid (AA), which is converted to a variety of proinflammatory eicosanoids.[3] Therefore, inhibiting AA release is of great therapeutic relevance for the development of new anti-inflammatory drugs. In many cases the activity of GIVA cPLA$_2$ has been shown to be dependent on or linked to the activity of sPLA$_2$.[4–6] GVIA iPLA$_2$ appears to be the primary phospholipase for basal metabolic functions within the cell, and perhaps has additional functions in specific cell types; however its role in inflammation is still unclear.[1,2,7,8]

The various classes of intracellular and extracellular PLA$_2$ inhibitors have been summarized in recent review articles.[9–11] Our laboratories have developed a novel class of GIVA cPLA$_2$ inhibitors designed to contain the 2-oxoamide functionality and a free carboxyl group.[12–20] 2-Oxoamides based on γ-aminobutyric acid (compound AX006, Fig. 1) and the non-natural amino acids γ-(*S*)-norleucine or δ-(*S*)-norleucine (compounds AX062 and AX109, Fig. 1) are potent inhibitors of GIVA cPLA$_2$ presenting in vivo anti-inflammatory and analgesic activity.[12,13,17] In addition, the 2-oxoamide ethyl ester derivative AX048 (Fig. 1), which in vitro inhibits both GIVA cPLA$_2$ and GVIA iPLA$_2$, presents a potent anti-hyperalgesic effect.[15] Most recently, we have reported that an amide based on γ-(*R*)-norleucine is a selective inhibitor of GV sPLA$_2$.[20]

To extend our studies on the inhibition of phospholipase A_2 by 2-oxoamides, we synthesized a variety of 2-oxoamides based on dipeptides and pseudodipeptides and we studied their in vitro activity on three human PLA$_2$ classes: GIVA cPLA$_2$, GVIA iPLA$_2$ and GV sPLA$_2$. Furthermore, to further understand the role and specificity of 2-oxoamide inhibitors in cells, we studied the effect of various 2-oxoamides on arachidonic acid release from RAW 264.7 macrophages.

2. Results and discussion

2.1. Design of inhibitors

Dipeptides are considered to be δ-amino acid analogues, because of the distance between the N- and C-terminal groups

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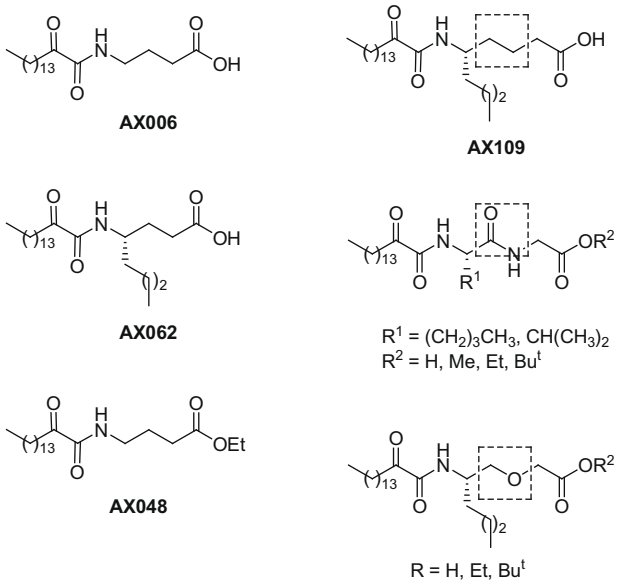


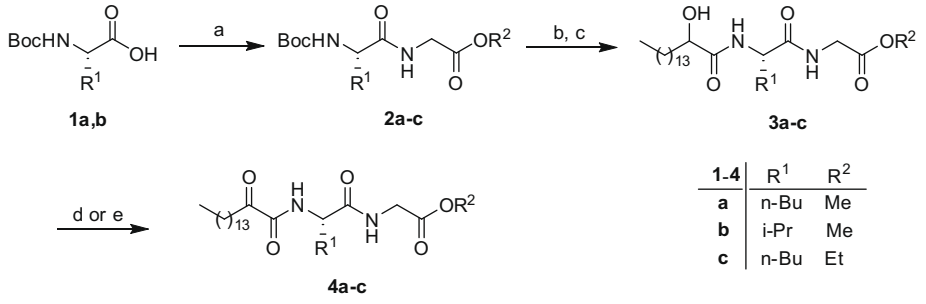
Figure 1. Known 2-oxoamide inhibitors and inhibitors designed for this work.

(Fig. 1). Ether pseudopeptide derivatives may be also considered δ-amino acid derivatives. Thus, in the present work we chose L-norleucine and L-valine as starting materials to synthesize a series of derivatives that contain the 2-oxoamide functionality and an amide group or ether group to replace the two methylenes of the δ-amino acid derivative. The replacement of the peptide bond with a suitable surrogate can increase the stability of peptides towards enzymatic hydrolysis, prolong the half-time of peptide action and improve the transport of peptides into cells.[21] In addition, using amide bond isosters is a convenient way to elucidate the role of the amide unit in protein–ligand interactions and thus, to understand the structure–biological activity relationship. The methylene ether group constitutes an interesting amide bond surrogate, since the calculated $C^i_\alpha - C^{i+1}_\alpha$ distance of ψ[CH_2O] pseudodipeptides (3.7 Å) is almost the same as that in a dipeptide (3.8 Å).[22] In the past, ether pseudopeptides have been studied such as renin[23] and cAMP-dependent protein kinase[24] inhibitors, as agonists of substance P,[25] and as analogues of the antidiuretic drug desmopressin.[26]

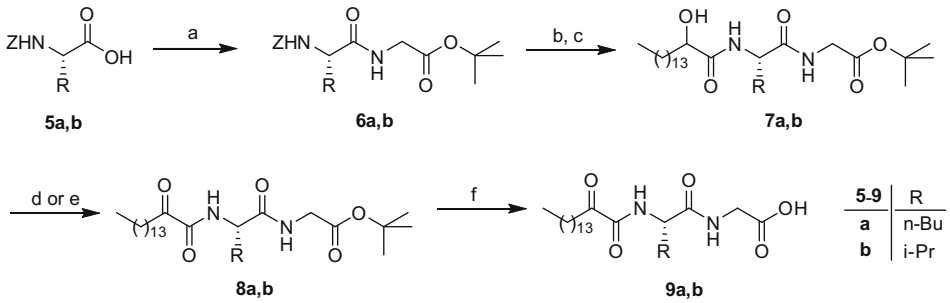
2.2. Synthesis of inhibitors

N-Protected L-norleucine (**1a**) and L-valine (**1b**) were coupled with methyl and ethyl glycinate using 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide (WSCI)[27] as a condensing agent in the presence of 1-hydroxybenzotriazole (HOBt) (Scheme 1). Removal of the Boc group, followed by coupling with 2-hydroxyhexadecanoic acid yielded 2-hydroxyamides **3a–c**, which were oxidized either by the Dess–Martin method[28] or by the NaOCl/AcNH-TEMPO method[29,30] to the target compounds **4a–c**. Following similar reactions, *tert*-butyl ester derivatives **8a**,**b** were prepared as depicted in Scheme 2, starting from Z-protected norleucine and valine. 2-Oxoamides **9a**,**b**, containing a free carboxyl group, were obtained by treatment of **8a**,**b** with trifluoroacetic acid.

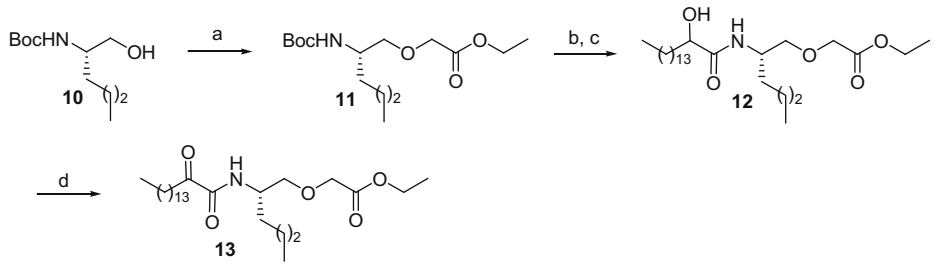
The synthesis of pseudopeptide derivatives is described in Schemes 3 and 4. Boc-L-norleucinol (**10**), obtained by reduction of Boc-L-norleucine,[31,32] reacted with ethyl bromoacetate using sodium hydride in the presence of 18-crown-6[22] to produce ether **11** (Scheme 3). Removal of Boc group, followed by coupling and oxidation yielded 2-oxoamide **13**. Pseudodipeptide derivatives **17** and **18** were synthesized by similar procedures (Scheme 4). However, for the synthesis of *tert*-butyl ester derivative the reaction of *Z*-L-



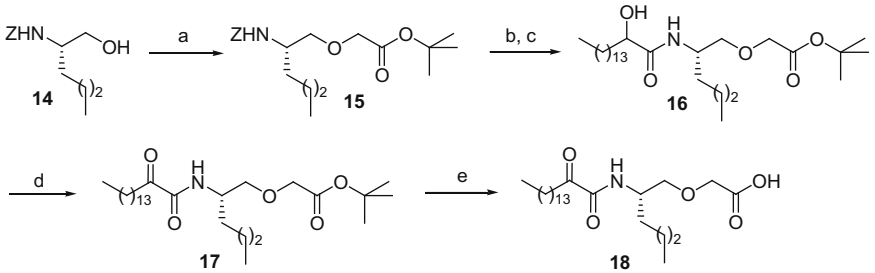
Scheme 1. Reagents and conditions: (a) HCl·H-Gly-OR^2, Et_3N, WSCI, HOBt, CH_2Cl_2; (b) 4 N HCl/Et_2O; (c) $CH_3(CH_2)_{13}CHOHCOOH$, Et_3N, WSCI, HOBt, CH_2Cl_2; (d) Dess–Martin reagent, CH_2Cl_2; (e) NaOCl, AcNH-TEMPO, NaBr, $NaHCO_3$, EtOAc/$PhCH_3$/H_2O 3:3:0.5, 0 °C.



Scheme 2. Reagents and conditions: (a) HCl·H-Gly-OBu^t, Et_3N, WSCI, HOBt, CH_2Cl_2; (b) H_2, 10% Pd/C, EtOH; (c) $CH_3(CH_2)_{13}CHOHCOOH$, Et_3N, WSCI, HOBt, CH_2Cl_2; (d) Dess–Martin reagent, CH_2Cl_2; (e) NaOCl, AcNH-TEMPO, NaBr, $NaHCO_3$, EtOAc/$PhCH_3$/H_2O 3:3:0.5, 0 °C; (f) 50% TFA/CH_2Cl_2.



Scheme 3. Reagents: (a) $BrCH_2COOEt$, NaH, 18-crown-6, THF; (b) 4 N HCl/Et_2O; (c) $CH_3(CH_2)_{13}CHOHCOOH$, Et_3N, WSCI, HOBt, CH_2Cl_2; (d) Dess–Martin reagent, CH_2Cl_2.



Scheme 4. Reagents: (a) $BrCH_2COOBu^t$, Bu_4NHSO_4, 50% NaOH, C_6H_6; (b) H_2, 10% Pd/C, EtOH; (c) $CH_3(CH_2)_{13}CHOHCOOH$, Et_3N, WSCI, HOBt, CH_2Cl_2; (d) Dess–Martin reagent, CH_2Cl_2; (e) 50% TFA/CH_2Cl_2.

norleucinol with *tert*-butyl bromoacetate was carried out under phase transfer conditions.[33,34]

2.3. In vitro inhibition of GIVA cPLA$_2$, GVIA iPLA$_2$ and GV sPLA$_2$

All the inhibitors synthesized in this work were tested for inhibition of GIVA cPLA$_2$, GVIA iPLA$_2$ and GV sPLA$_2$ using mixed micellar assays. Details for the assays have been published previously.[12,13,16,17] The results of the inhibition are presented in Table 1 using either percent inhibition or $X_I(50)$ values. Initially, the percent inhibition of each PLA$_2$ at 0.091 mole fraction of inhibitor was determined. $X_I(50)$ values were estimated when the percent inhibition was higher than 90%. The $X_I(50)$ is the mole fraction of the inhibitor in the total substrate interface required to inhibit the enzyme by 50%. Data for the reference 2-oxoamide inhibitors AX006 and AX048 are included in Table 1 for comparison.

Dipeptide-based 2-oxoamides **9a** and **9b** containing a free carboxyl group inhibited GIVA cPLA$_2$ without affecting the activity of the other intracellular enzyme GVIA iPLA$_2$. This observation is in full agreement with our previous report, that amino acid-based 2-oxoamides containing a free carboxyl group are selective inhibitors of GIVA cPLA$_2$.[16,17]

Methyl ester **4a** based on dipeptide Nle-Gly and *tert*-butyl ester **8b** based on dipeptide Val-Gly, inhibited both GIVA cPLA$_2$ and GVIA iPLA$_2$, showing a small preference for GIVA cPLA$_2$. The methyl ester **4b** based on dipeptide Val-Gly lost the activity against both GIVA cPLA$_2$ and GVIA iPLA$_2$. To the contrary, the *tert*-butyl ester **8a** and the ethyl ester **4c**, both based on Nle-Gly dipeptide presented potent inhibition of GVIA iPLA$_2$ with $X_I(50)$ values of 0.011 and 0.020, respectively. At the same time, both **8a** and **4c** inhibited very weakly GIVA cPLA$_2$ and GV sPLA$_2$.

A bioisosteric replacement of the amide bond in compounds **4c**, **8a** and **9a** by an ether group, resulted to the structurally related compounds **13**, **17** and **18**. Pseudodipeptide derivative **18**, selectively inhibited GIVA cPLA$_2$, as expected, due to the free carboxyl group. This derivative is the most potent inhibitor of GIVA cPLA$_2$ [$X_I(50)$ 0.017] identified in the present work and its potency is comparable to that of the reference 2-oxoamide inhibitor AX006 [$X_I(50)$ 0.024].[15] Interestingly, both ethyl ester **13** and *tert*-butyl ester **17** preferentially inhibited GVIA iPLA$_2$. In particular, derivative **13** is a potent inhibitor of GVIA iPLA$_2$ with a $X_I(50)$ value of 0.017, while high mole fraction (0.091) of the inhibitor caused only 52% inhibition of GIVA cPLA$_2$.

Among the compounds tested in this study, only two esters (compounds **8b** and **13**) were able to reduce GV sPLA$_2$ activity (around 80% inhibition at 0.091 mole fraction). GV sPLA$_2$ utilizes a catalytic histidine to activate a water molecule as the nucleophile in phospholipid hydrolysis. Although there is no serine nucleophile in GV sPLA$_2$, the 2-oxoamides may resemble the substrate phospholipids or the transition state such that they would bind to the GV sPLA$_2$ active site and inhibit the enzyme.

Both the intracellular enzymes GIVA cPLA$_2$ and GVIA iPLA$_2$ share the same catalytic mechanism using active site serine as the nucleophilic residue. Thus, compounds designed to inhibit GIVA cPLA$_2$, may show cross reactivity with GVIA iPLA$_2$. Very interestingly, ethyl and *tert*-butyl esters **4c**, **8a**, **13** and **17**, based on Nle-Gly or the corresponding ether pseudodipeptide preferentially inhibit GVIA iPLA$_2$. Up to now, we have shown that 2-oxoamide esters may inhibit both GIVA cPLA$_2$ and GVIA iPLA$_2$ either with similar potency or with a preference for GIVA cPLA$_2$.[16] However, we identified for the first time in the course of the present work several 2-oxoamides, such as **8a** or **13**, which show higher inhibition against GVIA iPLA$_2$ than GIVA cPLA$_2$. The dose-response curves of the inhibition of GVIA iPLA$_2$ by these inhibitors are shown in Figure 2. It seems that the replacement of the amino acid unit of a 2-oxoamide inhibitor by a dipeptide unit may shift the selectivity in favour of GVIA iPLA$_2$. Since there is a lack of potent and selective inhibitors for GVIA iPLA$_2$,[35] the findings of the present study may help in designing 2-oxoamides presenting selectivity for this particular PLA$_2$.

2.4. Ex vivo inhibition of cellular arachidonic acid release

Arachidonic acid (AA) is the ideal metabolic indicator of GIVA cPLA$_2$ activity, since it is the product of GIVA cPLA$_2$-mediated

Table 1
Inhibition of PLA_2 by 2-oxoamides based on dipeptides and pseudodipeptides[a]

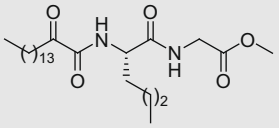
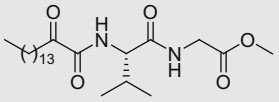
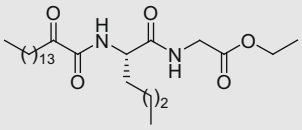
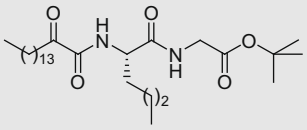
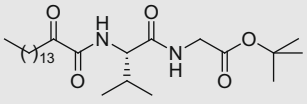
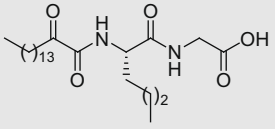
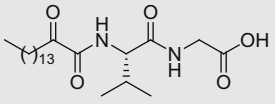
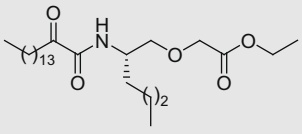
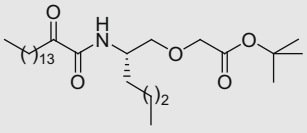
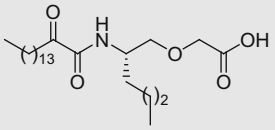
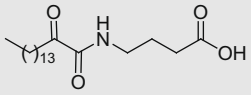
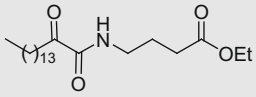
Compound	Structure	GIVA $cPLA_2$		GVIA $iPLA_2$		GV $sPLA_2$	
		$X_i(50)$	% Inhibition	$X_i(50)$	% Inhibition	$X_i(50)$	% Inhibition
4a		0.021 ± 0.009		0.045 ± 0.018			65
4b			25		N.D.[a]		N.D.[a]
4c			73	0.020 ± 0.002			63
8a			72	0.011 ± 0.001			59
8b		0.028 ± 0.015		0.044 ± 0.025			78
9a		0.035 ± 0.014			N.D.[a]		N.D.[a]
9b		0.061 ± 0.017			N.D.[a]		N.D.[a]
13			52	0.017 ± 0.002			81
17			44		81		57
18		0.017 ± 0.002			N.D.[a]		N.D.[a]

Table 1 (*continued*)

Compound	Structure	GIVA cPLA$_2$		GVIA iPLA$_2$		GV sPLA$_2$	
		X_i(50)	% Inhibition	X_i(50)	% Inhibition	X_i(50)	% Inhibition
AX006		0.024 ± 0.015[b]			N.D.[a,b]		
AX048		0.022 ± 0.009[b]		0.027 ± 0.009[b]			

[a] Average percent inhibition and standard error (n = 3) reported for each compound at 0.091 mole fraction. N.D. signifies compounds with less than 25% inhibition (or no detectable inhibition).
[b] Data taken from Ref. 15.

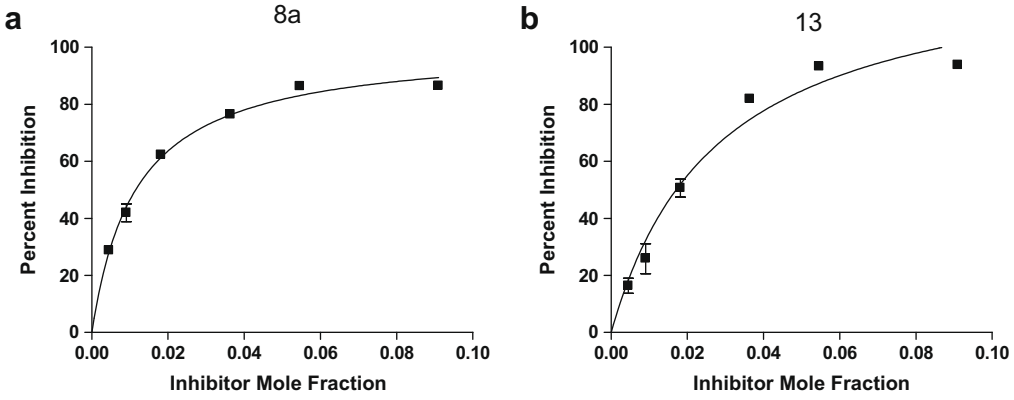


Figure 2. Dose–response curves for 2-oxoamide inhibitors of GVIA iPLA$_2$. Inhibition of the activity of human GVIA iPLA$_2$ by inhibitors (a) **8a** and (b) **13** was tested on mixed-micelles containing 100 μM PAPC and 400 μM Triton X-100. Inhibition curves were generated by Graphpad Prism using a non-linear regression (one-site binding model—hyperbola) to calculate the reported X_i(50) values.

catalysis. In addition to some of the new 2-oxoamides based on dipeptides and pseudodipeptides that were synthesized in this work, a series of potent 2-oxoamide in vitro inhibitors of GIVA cPLA$_2$, previously reported by us,[12,13,16,17,19,20] were further tested in a cellular ex vivo system to investigate whether they reduced cellular AA production. For this purpose, RAW 264.7 macrophages were preincubated with 25 μM concentrations of the inhibitor prior to treatment of the cells with Kdo$_2$-Lipid A. Subsequently, AA release was quantitated from the supernatants.

The results of our studies for 21 2-oxoamides are summarized in Table 2. Note that some of the compounds led to an actual increase or activation of AA release which is not unusual in this kind of assay due to detergent or other non-specific effects. Those compounds giving an activation of AA release were not evaluated further. Compounds **18**, **19**,[12] **20**,[12] **25**, **26**, **30**[16] and **35**,[17] which contain a free carboxyl group and are potent and selective inhibitors of GIVA cPLA$_2$, induce a significant reduction of AA release. Derivatives **26** and **30**, based on γ-norleucine, cause the highest inhibition percentage in AA release. Both strongly inhibit GIVA cPLA$_2$. Comparison between compounds **20** and **26** indicates the importance of the long aliphatic chain. Compound **19**, based on γ-aminobutyric acid and compound **25**, based on γ-leucine, cause significant inhibition, though to a lesser extent, of AA production. Moderate inhibitors of GIVA cPLA$_2$ result to a lower inhibition of AA release (compounds **21**,[17] **28**[20]). Compound **35**,[17] a δ-norleucine derivative, and compound **18**, based on an pseudodipeptide, which also can be considered as a δ-amino acid analogue, lead to a considerable AA reduction in cells. Both molecules are strong inhibitors of GIVA cPLA$_2$.

Although production of arachidonic acid can be catalyzed by other phospholipases as well, such as GVIA iPLA$_2$ and GV sPLA$_2$, most of the non-selective inhibitors of the three phospholipases tested here, did not show a significant inhibitory effect on AA release. Compounds **23**,[17] **24**,[15] **27**,[20] **29**,[16] **31**[20] and **36**,[20] carry an esterified carboxyl group. In compound **32**,[19] the ester group has been replaced by a sulfonamide group. They all inhibit GIVA cPLA$_2$ to a high extent, though not selectively, since they inhibit also both GVIA iPLA$_2$ and GV sPLA$_2$.

Dipeptide inhibitor **8b** reduced the cellular release of AA by 30%, while derivative **18** reduced AA production by 68%. Interestingly, the 2-oxoamide inhibitor **18**, which contains a free carboxyl group, leads to more than double the AA release inhibition, indicating the importance of selectivity of an inhibitor against GIVA cPLA$_2$.

2.5. IC$_{50}$ values for arachidonic acid inhibition

Dose–response curves were measured for the seven synthetic inhibitors that reduced AA release the most (see Table 2) in order to calculate the IC$_{50}$ values. All of these contained free carboxylic acids and were specific for GIVA cPLA$_2$. The IC$_{50}$ values for these seven compounds are summarized in Table 3. The individual dose–response curve for the inhibition of AA release from RAW 264.7 cells stimulated with Kdo$_2$-Lipid A in the presence of **18** is depicted

Table 2
Effect of 2-oxoamide inhibitors on Kdo_2-Lipid A stimulated AA release from RAW 264.7 macrophages

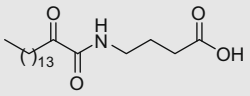
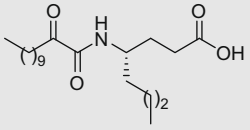
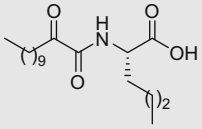
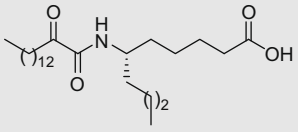
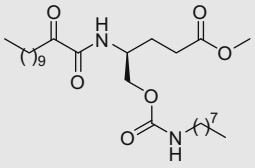
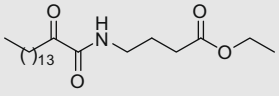
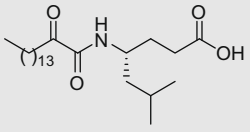
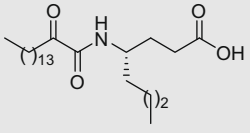
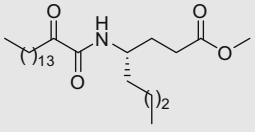
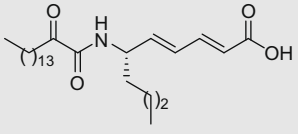
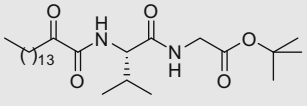
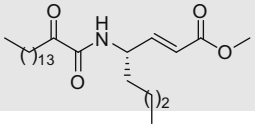
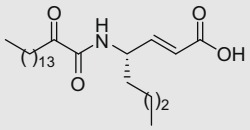
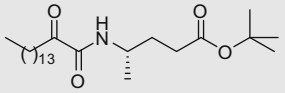
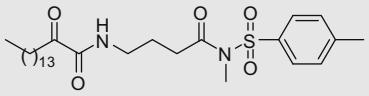
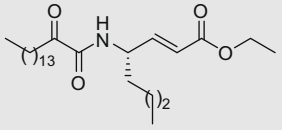
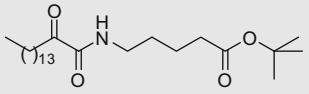
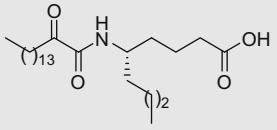
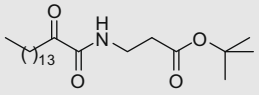
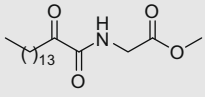
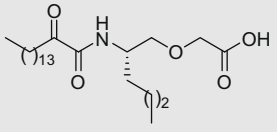
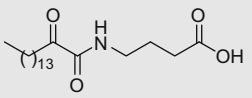
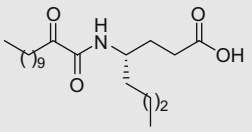
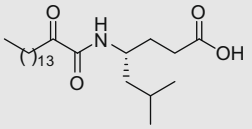
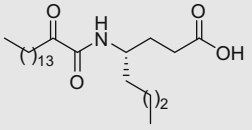
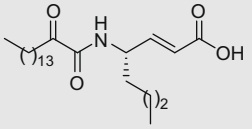
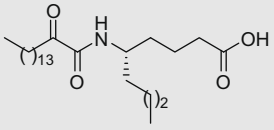
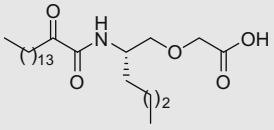
Compound	Structure	AA release inhibition (%)
19		52 ± 5
20		56 ± 6
21		27 ± 6
22		Activation
23		Activation
24		Activation
25		62 ± 5
26		79 ± 0.5
27		Activation
28		6 ± 10
8b		30 ± 2
29		Activation

Table 2 (*continued*)

Compound	Structure	AA release inhibition (%)
30		74 ± 8
31		Activation
32		Activation
33		16 ± 6
34		11 ± 6
35		59 ± 4
36		Activation
37		30 ± 2
18		68 ± 4

in Figure 3. Inhibitors **19**, **25**, **26** and **30** all display IC_{50} values of approximately 25 μM whereas **20**, **35** and **18** display values of 10, 7 and 2 μM, respectively. Interestingly, inhibitors **35** and **18**, which contain the carboxylic acid functional group spaced at the δ position relative to the 2-oxoamide moiety, demonstrated the lowest IC_{50} values at 7 and 2 μM, respectively. In contrast, the five γ linked inhibitors, **19**, **20**, **25**, **26** and **30**, all displayed higher IC_{50} values at 25, 10, 25, 25 and 25 μM. These data suggest that inhibitors with δ structural spacing display more effective AA release inhibition than γ structural spacing within this inflammatory cellular model. Of course, this specificity may relate to the ability of the compound to be taken up by the cell and other factors, but these results demonstrate that the oxoamides are able to inhibit within the cellular milieu.

Table 3
IC_{50} values for the inhibition of arachidonic acid release

Compound	Structure	IC_{50} (μM)
19		25
20		10
25		25
26		25
30		25
35		7
18		2

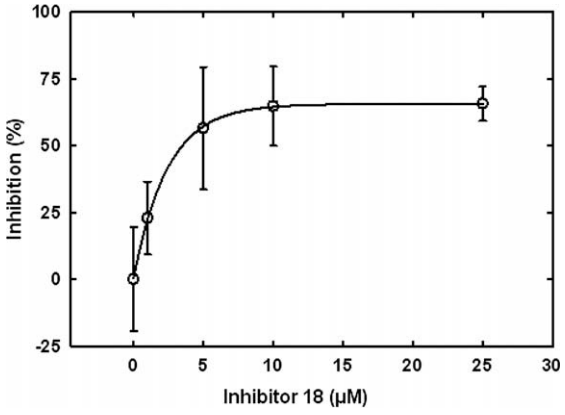


Figure 3. Dose–response curve for the inhibition of AA release from RAW 264.7 cells by inhibitor **18**. RAW 264.7 cells were preincubated with the indicated concentrations of **18** prior to stimulation with Kdo_2-Lipid A and AA quantitation. The data is graphically expressed in terms of percent inhibition of AA release as a function of the concentration of **18**. The curve is a fit of the data to a non-linear regression function using the sigma plot program. Based on the maximal inhibition observed in this assay, half maximal inhibition occurs at about 2 μM of **18**.

3. Conclusions

In conclusion, we have synthesized new 2-oxoamides based on dipeptides and pseudodipeptides and we have confirmed that derivatives containing a free carboxyl group are selective GIVA $cPLA_2$ inhibitors and also preferentially inhibit AA release intracellularly. 2-Oxoamides ethyl and *tert*-butyl esters, based on the dipeptide Nle-Gly or the corresponding ether pseudodipeptide preferentially inhibit the other major intracellular enzyme GVIA $iPLA_2$. Three of them are potent inhibitors of GVIA $iPLA_2$ with $X_I(50)$ values between 0.011 and 0.020. We demonstrated that selective GIVA $cPLA_2$ inhibitors inhibit AA release in RAW 264.7 macrophages and a 2-oxoamide based on a pseudodipeptide inhibits its AA release with an IC_{50} value of 2 μM.

4. Experimental

4.1. General

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 MHz) spectrometer. All amino acid derivatives were purchased from Fluka Chemical Co. Electron spray ionization (ESI) mass spectra were recorded on a Finnigan, Surveyor MSQ Plus spectrometer. TLC plates (Silica Gel 60 F254) and Silica Gel 60 (70–230 or 230–400 mesh) for column chromatography were purchased from Merck. Visualization of spots was effected with UV light and/or phosphomolybdic acid and/or ninhydrin, both in EtOH stain. THF was dried by standard procedures and stored over molecular sieves or Na. All other solvents and chemicals were reagent grade and used without further purification. Inhibitors **25** and **26** were prepared according to procedures described in the literature.[13,17]

4.2. Synthesis of 2-oxoamide inhibitors

4.2.1. General method for the coupling of 2-hydroxy-hexadecanoic acid with amino components

To a stirred solution of 2-hydroxy-hexadecanoic acid (0.27 g, 1.0 mmol) and hydrochloride amino component (1.0 mmol) in CH_2Cl_2 (10 mL), Et_3N (0.3 mL, 2.2 mmol) and subsequently 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (WSCI) (0.21 g, 1.1 mmol) and 1-hydroxybenzotriazole (HOBt) (0.14 g, 1.0 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 $CHCl_3$–$CHCl_3$/MeOH 99:1 as eluent.

4.2.1.1. Methyl 2-((*S*)-2-(2-hydroxyhexadecanamido)hexanamido)acetate (mixture of diastereomers) (3a). Yield 68%; white solid; mp 78–80 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.53–7.45 (m, 1H), 7.40 (d, J = 8.6 Hz, 1H), 4.59–4.43 (m, 1H), 4.15–4.06 (m,1H), 4.02–3.94 (m, 2H), 3.71 (s, 3H), 1.89–1.53 (m, 4H), 1.23 (br s, 28H), 0.98–0.80 (m, 6H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 175.1 (174.8), 172.7 (172.5), 170.1 (170.0), 72.0 (71.9), 52.4 (52.7), 52.2, 41.1, 34.8 (34.6), 32.0 (31.8), 29.6, 29.5, 29.3, 27.6, 27.5, 25.0, 22.6, 22.3, 22.2, 14.0, 13.8. Anal. Calcd for $C_{25}H_{48}N_2O_5$: C, 65.75; H, 10.59; N, 6.13. Found: C, 65.49; H, 10.78; N, 6.01.

4.2.1.2. Methyl 2-((*S*)-2-(2-hydroxyhexadecanamido)-3-methylbutanamido)acetate (mixture of diastereomers) (3b). Yield 73%; mp 109–111 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.34–7.15 (m, 2H), 4.43–4.27 (m, 1H), 4.10–3.96 (m, 3H), 3.75 (s, 3H), 2.24–2.08 (m, 1H), 1.87–1.63 (m, 2H), 1.26 (br s, 24H), 1.05–0.91 (m, 6H), 0.88 (t, J = 6.6 Hz, 3H). Anal. Calcd for $C_{24}H_{46}N_2O_5$: C, 65.12; H, 10.47; N, 6.33. Found: C, 64.98; H, 10.68; N, 6.18.

4.2.1.3. Ethyl 2-((*S*)-2-(2-hydroxyhexadecanamido)hexanamido)acetate (mixture of diastereomers) (3c). Yield 64%; white solid; mp 77–79 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.31–7.07 (m, 2H), 4.59–4.42 (m, 1H), 4.25–4.06 (m, 3H), 4.02–3.95 (m, 2H), 1.97–1.51 (m, 4H), 1.24 (br s, 31H), 0.97–0.80 (m, 6H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 174.9 (174.5), 172.4 (172.1), 169.7 (169.5), 72.1 (72.0), 61.5, 52.5 (52.7), 41.3, 34.6 (34.8), 31.9, 29.7, 29.4, 29.3, 27.6, 25.0, 22.7, 22.3, 14.1, 13.9. Anal. Calcd for $C_{26}H_{50}N_2O_5$: C, 66.35; H, 10.71; N, 5.95. Found: C, 66.19; H, 10.99; N, 5.89.

4.2.1.4. *tert*-Butyl 2-((*S*)-2-(2-hydroxyhexadecanamido)hexanamido)acetate (mixture of diastereomers) (7a). Yield 69%; white solid; mp 50–52 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.37–7.26 (m, 1H), 7.23–7.08 (m, 1H), 4.58–4.45 (m, 1H), 4.15–4.03 (m, 1H), 3.97–3.81 (m, 2H), 1.98–1.52 (m, 4H), 1.44 (s, 9H), 1.24 (br s, 28H), 0.98–0.79 (m, 6H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 174.9 (174.6), 172.4 (172.1), 168.6 (168.7), 82.2, 72.1 (72.0), 52.9 (52.5), 42.0, 34.8 (34.6), 32.1, 31.8, 29.6, 29.5, 29.3, 27.9, 27.6, 27.5, 25.0, 22.6, 22.3, 22.2, 14.0, 13.8. Anal. Calcd for $C_{28}H_{54}N_2O_5$: C, 67.43; H, 10.91; N, 5.62. Found: C, 67.28; H, 11.08; N, 5.47.

4.2.1.5. *tert*-Butyl 2-((*S*)-2-(2-hydroxyhexadecanamido)-3-methylbutanamido)acetate (mixture of diastereomers) (7b). Yield 81%; white solid; mp 81–83 °C; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.25 (d, J = 8.8 Hz, ½H), 7.16 (d, J = 8.8 Hz, ½H), 6.96 (t, J = 4.8 Hz, ½H), 6.88 (t, J = 4.8 Hz, ½H), 4.34–4.22 (m, 1H), 4.18–4.03 (m, 1H), 3.92–3.81 (m, 2H), 2.23–2.05 (m, 1H), 1.84–1.48 (m, 2H), 1.42 (s, 9H), 1.21 (br s, 24H), 0.93 (t, J = 5.8 Hz, 6H), 0.84 (t, J = 6.6 Hz, 3H); ^{13}C (50 MHz, $CDCl_3$) NMR δ 174.7 (175.0), 171.7 (171.4), 168.8 (168.6), 82.4, 72.3 (72.0), 58.1 (57.9), 42.0, 34.9 (34.6), 31.9, 30.8 (30.7), 29.7, 29.6, 29.4, 29.3, 28.0, 25.0, 22.6, 19.3, 18.1, 14.1. Anal. Calcd for $C_{27}H_{52}N_2O_5$: C, 66.90; H, 10.81; N, 5.78. Found: C, 66.65; H, 10.98; H, 5.62.

4.2.1.6. Ethyl 2-((*S*)-2-(2-hydroxyhexadecanamido)hexyloxy) acetate (mixture of diastereomers) (12). Yield 69%; waxy white solid; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.03–6.92 (m, 1H), 4.25–3.95 (m, 6H), 3.65–3.42 (m, 2H), 1.83–1.53 (m, 4H), 1.25 (br s, 31H), 0.95–1.79 (m, 6H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 174.2 (173.9), 170.8 (170.7), 73.2 (73.1), 72.2 (71.8), 68.0 (68.1), 61.0, 48.5 (48.8), 34.9 (34.7), 31.8, 31.0, 29.6, 29.5, 29.4, 29.3, 28.1, 24.9, 24.8, 22.6, 22.5, 14.0, 13.9. Anal. Calcd for $C_{26}H_{51}NO_5$: C, 68.23; H, 11.23; N, 3.06. Found: C, 68.04; H, 11.34; N, 2.91.

4.2.1.7. *tert*-Butyl 2-((*S*)-2-(2-hydroxyhexadecanamido)hexyloxy)acetate (mixture of diastereomers) (16). Yield 77%; oil; ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.05 (d, J = 8.8 Hz, 1H), 4.17–3.96 (m, 2H), 3.93 (s, 2H), 3.73 (br s, 1H), 3.57 (dd, J_1 = 9.6 Hz, J_2 = 3.6 Hz, 1H), 3.45 (dd, J_1 = 8.8 Hz, J_2 = 3.6 Hz, 1H), 1.90–1.55 (m, 4H), 1.45 (s, 9H), 1.23 (br s, 28H), 0.98–1.78 (m, 6H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 174.2 (174.0), 170.1, 82.0, 73.2, 72.2 (71.7), 68.4 (68.6), 48.7 (48.9), 34.7 (35.0), 31.9, 31.0, 29.6, 29.5, 29.3, 28.2, 28.0, 24.9, 24.8, 22.6, 22.5, 14.0, 13.9. Anal. Calcd for $C_{28}H_{55}NO_5$: C, 69.23; H, 11.41; N, 2.88. Found: C, 69.01; H, 11.59; N, 2.73.

4.2.2. General method for the oxidation of 2-hydroxy-amides. Method A

To a solution of 2-hydroxy-amide (1 mmol) in dry CH_2Cl_2 (10 mL) Dess–Martin periodinane was added (0.64 g, 1.5 mmol) and the mixture was stirred for 1 h at room temperature. The organic solution was washed with 10% aqueous $NaHCO_3$, dried over Na_2SO_4 and the organic solvent was evaporated under reduced pressure. The residue was purified by column-chromatography using $CHCl_3$ as eluent.

4.2.2.1. (*S*)-Methyl 2-(2-(2-oxohexadecanamido)hexanamido)acetate (4a). Yield 85%; white solid; mp 65–67 °C; $[\alpha]_D$ = −21.8 (*c* 0.5 $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.50 (d, J = 8.4 Hz, 1H), 6.74 (t, J = 5.4 Hz, 1H), 4.49–4.37 (m, 1H), 4.17–3.95 (m, 2H), 3.75 (s, 3H), 2.89 (t, J = 7.6 Hz, 2H), 2.05–1.47 (m, 4H), 1.24 (br s, 26H), 0.97–0.81 (m, 6H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 198.3, 170.9, 170.0, 160.0, 53.0, 52.4, 41.1, 36.8, 31.9, 31.8, 29.6, 29.5, 29.4, 29.3, 29.0, 27.4, 23.0, 22.6, 22.3, 14.1, 13.8; MS (ESI): *m/z* (%): 477 (77) $[M+Na]^+$. Anal. Calcd for $C_{25}H_{46}N_2O_5$: C, 66.04; H, 10.20; N, 6.16. Found: C, 66.19; H, 10.13; N, 6.21.

4.2.2.2. (*S*)-Ethyl 2-(2-(2-oxohexadecanamido)hexanamido)acetate (4c). Yield 76%; white solid; mp 63–65 °C; $[\alpha]_D$ = −20.8 (*c* 0.5 $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.51 (d, J = 8.4 Hz, 1H), 6.76 (t, J = 5.2, 1H), 4.50–4.36 (m, 1H), 4.19 (q, J = 7 Hz, 2H), 4.12–3.91 (m, 2H), 2.88 (t, J = 7.4 Hz, 2H), 2.05–1.45 (m, 4H), 1.26 (br s, 29H), 0.95–0.80 (m, 6H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 198.3, 170.9, 169.5, 160.1, 61.6, 53.0, 41.3, 36.8, 32.0, 31.9, 29.6, 29.5, 29.4, 29.3, 29.0, 27.5, 23.0, 22.6, 22.3, 14.1, 13.8; MS (ESI): *m/z* (%): 491 (100) $[M+Na]^+$, 469 (55) $[M+]^+$. Anal. Calcd for $C_{26}H_{48}N_2O_5$: C, 66.63; H, 10.32; N, 5.98. Found: C, 66.58; H, 10.39; N, 5.91.

4.2.2.3. (*S*)-*tert*-Butyl 2-(2-(2-oxohexadecanamido)hexanamido)acetate (8a). Yield 85%; white solid; mp 38–39 °C; $[\alpha]_D$ = −17.2 (*c* 0.5 $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.47 (d, J = 8.4 Hz, 1H), 6.57 (t, J = 5 Hz, 1H), 4.48–4.35 (m, 1H), 4.03–3.80 (m, 2H), 2.89 (t, J = 8 Hz, 2H), 2.02–1.52 (m, 4H), 1.45 (s, 9H), 1.24 (br s, 26H), 0.97–0.79 (m, 6H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 198.3, 170.7, 168.5, 160.1, 82.4, 53.1, 42.0, 36.8, 32.0, 31.9, 29.6, 29.5, 29.4, 29.3, 29.0, 28.0, 27.5, 23.1, 22.6, 22.3, 14.1, 13.8; MS (ESI): *m/z* (%): 519 (100) $[M+Na]^+$, 497 (32) $[M+H]^+$. Anal. Calcd for $C_{28}H_{52}N_2O_5$: C, 67.70; H, 10.55; N, 5.64. Found: C, 67.58; H, 10.73; N, 5.58.

4.2.2.4. (*S*)-Ethyl 2-(2-(2-oxohexadecanamido)hexyloxy)acetate (13). Yield 88%; white solid; mp 41–43 °C; $[\alpha]_D$ = −8.4 (*c* 0.5 $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.29 (d, J = 9.6 Hz, 1H), 4.21 (q, J = 7.4 Hz, 2H), 4.06 (s, 2H), 4.05–3.91 (m, 1H), 3.64 (dd, J_1 = 9.6 Hz, J_2 = 4.0 Hz, 1H), 3.52 (dd, J_1 = 9.4 Hz, J_2 = 3.8 Hz, 1H), 2.90 (t, J = 6.6 Hz, 2H), 1.71–1.48 (m, 4H), 1.24 (br s, 29H), 0.96–0.80 (m, 6H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 199.2, 170.3, 159.9, 72.5, 68.2, 60.9, 49.4, 36.8, 31.9, 30.9, 29.6, 29.5, 29.4, 29.3, 29.0, 28.0, 23.1, 22.6, 22.4, 14.1, 13.9; MS (ESI): *m/z* (%): 478 (100) $[M+Na]^+$. Anal. Calcd for $C_{26}H_{49}NO_5$: C, 68.53; H, 10.84; N, 3.07. Found: C, 68.65; H, 10.71; N, 3.12.

4.2.2.5. (*S*)-*tert*-Butyl 2-(2-(2-oxohexadecanamido)hexyloxy) acetate (17). Yield 93%; white solid; low mp; $[\alpha]_D$ = −10.8 (*c* 0.5 $CHCl_3$); ^{1}H NMR (200 MHz, $CDCl_3$) δ 7.26 (d, J = 8.8 Hz, 1H), 4.03–3.85 (m, 3H), 3.61 (dd, J_1 = 9.4 Hz, J_2 = 4.0 Hz, 1H), 3.49 (dd, J_1 = 9.4 Hz, J_2 = 4.0 Hz, 1H), 2.89 (t, J = 7.2 Hz, 2H), 1.69–1.48 (m, 4H), 1.45 (s, 9H), 1.23 (br s, 26H), 0.96–0.79 (m, 6H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 199.2, 169.4, 160.0, 81.7, 72.4, 68.7, 49.4, 36.7, 31.8, 31.0, 29.6, 29.5, 29.4, 29.3, 29.0, 28.0, 23.1, 22.6, 22.4, 14.0, 13.9; MS (ESI): *m/z* (%): 506 (57) $[M+Na]^+$. Anal. Calcd for $C_{28}H_{53}NO_5$: C, 69.52; H, 11.04; N, 2.90. Found: C, 69.57; H, 11.08; N, 2.83.

4.2.3. General method for the oxidation of 2-hydroxy-amides. Method B

To a solution of 2-hydroxy-amide (1.0 mmol) in a mixture of toluene (3 mL) and EtOAc (3 mL) a solution of NaBr (0.11 g, 1.1 mmol) in water (0.5 mL) was added followed by AcNH-TEMPO (2.2 mg, 0.01 mmol). To the resulting biphasic system, which was cooled at 0 °C, an aqueous solution of 0.35 M NaOCl (3.1 mL,

1.1 mmol) containing $NaHCO_3$ (0.25 g, 3 mmol) was added dropwise under vigorous stirring, at 0 °C over a period of 1 h. After the mixture had been stirred for a further 15 min at 0 °C, EtOAc (10 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 (10 mL) containing KI (0.04 g), 10% aqueous $Na_2S_2O_3$ (10 mL), and brine and dried over Na_2SO_4. The solvents were evaporated under reduced pressure and the residue was purified by column-chromatography using $CHCl_3$ as eluent.

4.2.3.1. (*S*)-Methyl 2-(3-methyl-2-(2-oxohexadecanamido)butanamido)acetate (4b). Yield 73%; white solid; mp 118–119 °C; $[\alpha]_D = -20.9$ (*c* 1 $CHCl_3$); ^1H NMR (200 MHz, $CDCl_3$) δ 7.46 (d, $J = 9.2$ Hz, 1H), 6.53–6.37 (m, 1H), 4.24 (dd, $J_1 = 6.6$ Hz, $J_2 = 9.2$ Hz, 1H), 4.05 (dd, $J_1 = 5.2$, $J_2 = 9.6$ Hz, 2H), 3.77 (s, 3H), 2.91 (t, $J = 7.4$ Hz, 2H), 2.22 (m, 1H), 1.61 (m, 2H), 1.26 (br s, 22H), 0.98 (t, $J = 5.8$ Hz, 6H), 0.88 (t, $J = 6.6$ Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 198.3, 170.9, 170.0, 160.2, 58.6, 52.5, 41.1, 36.8, 31.9, 31.0, 29.6, 29.4, 29.3, 29.0, 23.1, 22.7, 19.2, 18.0, 14.1. Anal. Calcd for $C_{24}H_{44}N_2O_5$: C, 65.42; H, 10.07; N, 6.36. Found: C, 65.55; H, 9.97; N, 6.41.

4.2.3.2. (*S*)-*tert*-Butyl 2-(3-methyl-2-(2-oxohexadecanamido) butanamido)acetate (8b). Yield 90%; white solid; mp 58–61 °C; $[\alpha]_D = -17.2$ (*c* 1 $CHCl_3$); ^1H NMR (200 MHz, $CDCl_3$) δ 7.50 (d, $J = 8.8$ Hz, 1H), 6.59–6.45 (m, 1H), 4.34–3.18 (m, 1H), 4.01 (dd, $J_1 = 18.2$ Hz, $J_2 = 5.4$ Hz, 1H), 3.85 (dd, $J_1 = 18.2$ Hz, $J_2 = 4.8$ Hz, 1H), 2.89 (t, $J = 6.8$ Hz, 2H), 2.28–2.19 (m, 1H), 1.64–1.48 (m, 2H), 1.45 (s, 9H), 1.24 (br s, 22H), 0.96 (t, $J = 5.8$ Hz, 6H), 0.87 (t, $J = 6.6$ Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 198.3, 170.1, 168.5, 160.2, 82.4, 58.5, 41.9, 36.8, 31.9, 31.1, 29.6, 29.5, 29.4, 29.3, 29.0, 28.0, 23.1, 22.6, 19.2, 18.0, 14.1; MS (FAB): *m/z* (%): 483 (24) $[M+H]^+$. Anal. Calcd for $C_{27}H_{50}N_2O_5$: C, 67.18; H, 10.44; N, 5.80. Found: C, 67.31; H, 10.39; N, 5.68.

4.2.4. General method for the cleavage of *tert*-butyl protecting group

A solution of the *tert*-butyl ester derivative (1 mmol) in 50% TFA/CH_2Cl_2 (10 mL) was stirred for 1 h at room temperature. The organic solvent was evaporated under reduced pressure. The residue was purified by recrystallization [EtOAc/petroleum ether (bp 40–60 °C)].

4.2.4.1. (*S*)-2-(2-(2-Oxohexadecanamido)hexanamido)acetic acid (9a). Yield 61%; colorless oil; ^1H NMR (200 MHz, $CDCl_3$) δ 7.66 (d, $J = 8.8$ Hz, 1H), 7.20–7.05 (m, 1H), 4.60–4.43 (m, 1H), 4.06 (d, $J = 3.6$ Hz, 2H), 2.88 (t, $J = 3.8$ Hz, 2H), 1.83–1.59 (m, 4H), 1.25 (br s, 26H), 0.89 (t, $J = 8.0$ Hz, 6H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 198.1, 172.6, 171.4, 160.1, 53.0, 41.4, 36.9, 32.1, 31.9, 29.6, 29.4, 29.3, 29.0, 27.5, 23.0, 22.7, 22.3, 14.1, 13.8; MS (ESI): *m/z* (%): 442 (100) $[M+H]^+$. Anal. Calcd for $C_{24}H_{44}N_2O_5$: C, 65.42; H, 10.07; N, 6.36. Found: C, 65.19; H, 10.32; N, 6.25.

4.2.4.2. (*S*)-2-(3-Methyl-2-(2-oxohexadecanamido)butanamido)acetic acid (9b). Yield 68%; white solid; mp 87–89 °C; $[\alpha]_D = -1.8$ (*c* 0.5 $CHCl_3$); ^1H NMR (200 MHz, $CDCl_3$) δ 7.71 (d, $J = 9$ Hz, 1H), 7.21–7.06 (m, 1H), 4.45–4.34 (m, 1H), 4.18–4.01 (m, 2H), 3.03–2.72 (m, 2H), 2.25–2.04 (m, 1H), 1.68–1.45 (m, 2H), 1.25 (br s, 22H), 0.97 (t, $J = 5.8$ Hz, 6H), 0.88 (t, $J = 6.6$ Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 198.0, 172.3, 170.9, 160.4, 58.6, 41.3, 36.9, 31.9, 31.1, 29.6, 29.4, 29.3, 29.0, 23.0, 22.7, 19.1, 18.1, 14.1; MS (ESI): *m/z* (%): 425 (100) $[M-H]^-$. Anal. Calcd for $C_{23}H_{42}N_2O_5$: C, 64.76; H, 9.92; N, 6.57. Found: C, 64.65; H, 9.87; N, 6.63.

4.2.4.3. (*S*)-2-(2-(2-Oxohexadecanamido)hexyloxy)acetic acid (18). Yield 88%; white solid; mp 64–66 °C; $[\alpha]_D = -5.2$ (*c* 0.5 $CHCl_3$); ^1H NMR (200 MHz, $CDCl_3$) δ 7.32 (d, $J = 8.8$ Hz, 1H), 4.14 (s, 2H), 4.07–3.95 (m, 1H), 3.66 (dd, $J_1 = 9.6$ Hz, $J_2 = 4.8$ Hz, 1H), 3.55 (dd, $J_1 = 9.6$ Hz, $J_2 = 3.6$ Hz, 1H), 2.90 (t, $J = 7.4$ Hz, 2H), 1.75–1.46 (m, 4H), 1.25 (br s, 26H), 0.98–0.80 (m, 6H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 199.1, 174.6, 160.2, 72.9, 67.8, 49.5, 36.8, 31.9, 30.8, 29.6, 29.4, 29.3, 29.0, 28.0, 23.2, 22.6, 22.4, 14.1, 13.9; MS (ESI): *m/z* (%): 450 (100) $[M+Na]^+$. Anal. Calcd for $C_{24}H_{45}NO_5$: C, 67.41, H, 10.61, N, 3.28. Found: C, 67.59, H, 10.64, N, 3.14.

4.2.5. General method for the synthesis of dipeptides

The dipeptides were prepared following the general method 4.2.1.

4.2.5.1. (*S*)-Methyl 2-(2-(*tert*-butoxycarbonylamino)hexanamido)acetate (2a)[38,39]. Yield 65%; white solid; mp 95–96 °C; $[\alpha]_D = -17.8$ (*c* 1 $CHCl_3$); ^1H NMR (200 MHz, $CDCl_3$) δ 6.85–6.73 (m, 1H), 5.12 (d, $J = 8.2$ Hz, 1H), 4.22–4.07 (m, 1H), 4.03 (d, $J = 5.6$ Hz, 2H), 3.74 (s, 3H), 1.93–1.72 (m, 1H), 1.69–1.48 (m, 1H), 1.43 (s, 9H), 1.39–1.21 (m, 4H), 0.88 (t, $J = 6.6$ Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.6, 170.1, 155.7, 80.0, 54.4, 52.3, 41.0, 32.2, 28.2, 27.6, 22.3, 13.9.

4.2.5.2. (*S*)-Methyl 2-(2-(*tert*-butoxycarbonylamino)-3-methylbutanamido)acetate (2b). Yield 73%; white solid; mp 104–106 °C; $[\alpha]_D = -15.7$ (*c* 1 $CHCl_3$); ^1H NMR (200 MHz, $CDCl_3$) δ 6.64 (m, 1H), 5.09 (d, $J = 7.6$ Hz, 1H), 4.06–3.96 (m, 3H), 3.76 (s, 3H), 2.19 (m, 1H), 1.42 (s, 9H), 0.99 (d, $J = 7.0$ Hz, 3H), 0.94 (d, $J = 7.0$ Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 171.9, 170.1, 155.7, 80.0, 59.7, 52.3, 41.0, 30.8, 28.2, 19.2, 18.0. Anal. Calcd for $C_{13}H_{24}N_2O_5$: C, 54.15; H, 8.39; N, 9.72. Found: C, 53.92; H, 8.52; N, 9.61.

4.2.5.3. (*S*)-Ethyl 2-(2-(*tert*-butoxycarbonylamino)hexanamido) acetate (2c)[40]. Yield 69%; oil; $[\alpha]_D = -14.8$ (*c* 1 $CHCl_3$); ^1H NMR (200 MHz, $CDCl_3$) δ 7.00–6.85 (m, 1H), 5.23 (d, $J = 7.2$ Hz, 1H), 4.22–4.05 (m, 3H), 4.02–3.93 (m, 2H), 1.91–1.69 (m, 1H), 1.66–1.45 (m, 1H), 1.40 (s, 9H), 1.35–1.18 (m, 7H), 0.86 (t, $J = 6.6$ Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 172.7, 169.6, 155.6, 79.8, 61.3, 54.3, 41.1, 32.3, 28.2, 27.5, 22.3, 14.0, 13.8.

4.2.5.4. (*S*)-*tert*-Butyl 2-(2-(benzyloxycarbonylamino)hexanamido)acetate (6a). Yield 64%; white solid; mp 80–82 °C; $[\alpha]_D = -8.4$ (*c* 0.5 $CHCl_3$); ^1H NMR (200 MHz, $CDCl_3$) δ 7.41–7.26 (m, 5H), 6.62–6.49 (m, 1H), 5.41 (d, $J = 8.0$ Hz, 1H), 5.12 (s, 2H), 4.29–4.11 (m, 1H), 3.92 (d, $J = 4.6$ Hz, 2H), 1.98–1.77 (m, 1H), 1.75–1.59 (m, 1H), 1.47 (s, 9H), 1.42–1.21 (m, 4H), 0.89 (t, $J = 7$ Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 171.9, 168.7, 156.1, 136.2, 128.5, 128.1, 128.0, 82.3, 67.0, 54.9, 42.0, 32.4, 28.0, 27.5, 22.3, 13.8. Anal. Calcd for $C_{20}H_{30}N_2O_5$: C, 63.47; H, 7.99; N, 7.40. Found: C, 63.28; H, 8.15; H, 7.28.

4.2.5.5. (*S*)-*tert*-Butyl 2-(2-(benzyloxycarbonylamino)-3-methyl-butanamido)acetate (6b). Yield 81%; white solid; mp 139–141 °C; $[\alpha]_D = -6.8$ (*c* 0.5 $CHCl_3$); ^1H NMR (200 MHz, $CDCl_3$) δ 7.42–7.28 (m, 5H), 6.47–6.35 (m, 1H), 5.39 (d, $J = 8.6$ Hz, 1H), 5.12 (s, 2H), 4.11–4.00 (m, 1H), 4.99–3.83 (m, 2H), 2.28–2.05 (m, 1H), 1.47 (s, 9H), 0.99 (d, $J = 7.0$ Hz, 3H), 0.94 (d, $J = 7.0$ Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 171.2, 168.6, 156.0, 136.1, 128.5, 128.2, 128.0, 82.4, 67.1, 60.3, 42.0, 31.0, 28.0, 19.2, 17.6. Anal. Calcd for $C_{19}H_{28}N_2O_5$: C, 62.62; H, 7.74; N, 7.69. Found: C, 62.39; H, 7.91; N, 7.55.

4.2.6. (*S*)-Ethyl 2-(2-(*tert*-butoxycarbonylamino) hexyloxy)acetate (11)

To as stirred solution of Boc-L-Nle-ol (0.50 g, 1.9 mmol) in dry THF (15 mL), cooled at 0 °C under nitrogen, NaH (0.05 g, 2.1 mmol) was added. The reaction mixture was stirred for 45 min at 0 °C, followed by addition of 18-crown-6 (0.25 g, 0.9 mmol) and subsequently a solution of ethyl bromoacetate (0.47 g, 2.8 mmol) in dry THF (4 mL) was added dropwise at 0 °C. The reaction mixture was stirred for 1 h at 0 °C and overnight at room temperature. The organic solvent was evaporated under reduced pressure and the residue was purified by column-chromatography using $CHCl_3$ as eluent. Yield 55%; oil; $[\alpha]_D = -13.9$ (*c* 1 $CHCl_3$); ^1H NMR (200 MHz, $CDCl_3$) δ 4.85–4.78 (m, 1H), 4.17 (q, $J = 7.4$ Hz, 2H), 4.03 (s, 2H), 3.72–3.58 (m, 1H), 3.56–3.41 (m, 2H), 1.62–1.21 (m, 18H), 0.85 (t, $J = 6.6$ Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 170.3, 155.6, 78.9, 73.3, 68.4, 60.7, 50.3, 31.5, 28.3, 28.1, 22.5, 14.1, 13.9. Anal. Calcd for $C_{15}H_{29}NO_5$: C, 59.38; H, 9.63; N, 4.62. Found: C, 59.16; H, 9.82; N, 4.51.

4.2.7. (*S*)-*tert*-Butyl 2-(2-(benzyloxycarbonylamino) hexyloxy)acetate (15)

To a stirred solution of *Z*-L-Nle-ol (0.20 g, 0.8 mmol) in benzene (0.8 mL), *tert*-butyl bromoacetate (0.47 g, 2.4 mmol) and subsequently aq NaOH 50% (0.8 mL) and the phase transfer catalyst Bu_4NHSO_4 (0.07 g, 0.2 mmol) were added. The reaction mixture was vigorously stirred for 2 h. EtOAc (10 mL) and H_2O (10 mL) were added and the aqueous layer was separated and extracted with EtOAc (2×10 mL). The combined organic layers were washed with brine and dried over Na_2SO_4. The organic solvent was evaporated under reduced pressure and the residue was purified by column-chromatography using petroleum ether (bp 40–60 °C)/EtOAc 8:2 as eluent. Yield 74%; oil; $[\alpha]_D = -12.2$ (*c* 1 $CHCl_3$); ^1H NMR (200 MHz, $CDCl_3$) δ 7.34–7.22 (m, 5H), 5.30 (d, $J = 8$ Hz, 1H), 5.06 (s, 2H), 3.91 (s, 2H), 3.80–3.62 (m, 1H), 3.55 (dd, $J_1 = 9.0$ Hz, $J_2 = 4.0$ Hz, 1H), 3.46 (dd, $J_1 = 9.2$ Hz, $J_2 = 4.2$ Hz, 1H), 1.62–1.45 (m, 2H), 1.43 (s, 9H), 1.37–1.21 (m, 4H), 0.85 (t, $J = 6.8$ Hz, 3H); ^{13}C NMR (50 MHz, $CDCl_3$) δ 169.5, 156.0, 136.6, 128.3, 128.0, 127.8, 81.5, 73.0, 68.7, 66.3, 50.9, 31.4, 28.0, 27.9, 22.4, 13.8. Anal. Calcd for $C_{20}H_{31}NO_5$: C, 65.73; H, 8.55; N, 3.83. Found: C, 65.52; H, 8.71; H, 3.70.

4.3. In vitro PLA_2 assays

Phospholipase A_2 activity was determined using the previously described modified Dole assay[12] with buffer and substrate conditions optimized for each enzyme as described previously:[12,13,16,17] (i) GIVA $cPLA_2$ substrate mixed-micelles were composed of 400 μM Triton X-100, 97 μM PAPC, 1.8 μM ^{14}C-labelled PAPC and 3 μM PIP_2 in buffer containing 100 mM HEPES pH 7.5, 90 μM $CaCl_2$, 2 mM DTT and 0.1 mg/ml BSA; (ii) GVI $iPLA_2$ substrate mixed-micelles were composed of either (a) 400 μM Triton X-100, 99 μM DPPC and 1.5 μM ^{14}C-labelled DPPC in buffer containing 200 mM HEPES pH 7.0, 1 mM ATP, 2 mM DTT and 0.1 mg/ml BSA or (b) 400 μM Triton X-100, 98.3 μM PAPC and 1.7 μM ^{14}C-labelled PAPC in buffer containing 100 mM HEPES pH 7.5, 2 mM ATP and 4 mM DTT and (iii) GV $sPLA_2$ substrate mixed-micelles were composed of 400 μM Triton X-100, 99 μM DPPC and 1.5 μM ^{14}C-labelled DPPC in buffer containing 50 mM Tris pH 8.0 and 5 mM $CaCl_2$.

4.4. In vitro PLA_2 inhibition studies

Initial screening of compounds at 0.091 mole fraction inhibitor in mixed-micelles was carried out. We considered compounds displaying 25% or less inhibition to have no inhibitory affect (designated N.D.). We report average percent inhibition (and standard error, $n = 3$) for compounds displaying more than 25% and less than 90% enzyme inhibition. If the percent inhibition was greater than 90%, we determined its $X_I(50)$ by plotting percent inhibition versus inhibitor molar fraction (7 points; typically 0.005–0.091 mole fraction). Inhibition curves were modeled in Graphpad Prism using either a linear (*x*, *y* intercept = 0) or non-linear regression (one-site binding model—hyperbola, BMAX = 100) to calculate the reported $X_I(50)$ and associated error values.

4.5. Inhibition of arachidonic acid release. RAW 264.7 cell culture and AA quantitation

RAW 264.7 macrophages were maintained in a humidified atmosphere at 37 °C with 5% CO_2, as described elsewhere.[36] The cells were cultured in DMEM media that was supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/mL penicillin, 100 μg/ml streptomycin, and non-essential amino acids. The cells were plated at a confluency of 2×10^6 cells per well in 6 well tissue culture plates and allowed to adhere overnight. Inhibitors were added to the medium 30 min before Kdo_2-Lipid A was added and the supernatants were collected at 1 h following stimulation. Subsequently, AA was extracted from the supernatants and quantitated by HPLC-MS, as described elsewhere.[36,37] AA release levels were normalized to pmol AA release per million cells though DNA quantitation using the Broad Range DNA Quant-Kit (Invitrogen). IC_{50} values were determined by estimating the concentration of inhibitor required for half maximal inhibition of the maximum inhibition observed with a given inhibitor.

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