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2-Aminohydroxamic acid derivatives as inhibitors of *Bacillus cereus* phosphatidylcholine preferred phospholipase C PC-PLC$_{Bc}$

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ABSTRACT

Phosphatidylcholine preferring phospholipase C (PC-PLC) is an important enzyme that plays a key role in a variety of cellular events and lipid homoeostases. *Bacillus cereus* phospholipase C (PC-PLC$_{Bc}$) has antigenic similarity with the elusive mammalian PC-PLC, which has not thus far been isolated and purified. Therefore the discovery of inhibitors of PC-PLC$_{Bc}$ is of current interest. Here, we describe the synthesis and biological evaluation of a new type of compounds inhibiting PC-PLC$_{Bc}$. These compounds have been designed by evolution of previously described 2-aminohydroxamic acid PC-PLC$_{Bc}$ inhibitors that block the enzyme by coordination of the zinc active site atoms present in PC-PLC$_{Bc}$ [Gonzalez-Roura, A.; Navarro, I.; Delgado, A.; Llebaria, A.; Casas, J. *Angew. Chem. Int. Ed.* **2004**, *43*, 862]. The new compounds maintain the zinc coordinating groups and possess an extra trimethylammonium function, linked to the hydroxyamide nitrogen by an alkyl chain, which is expected to mimic the trimethylammonium group of the phosphatidylcholine PC-PLC$_{Bc}$ substrates. Some of the compounds described inhibit the enzyme with IC$_{50}$'s in the low micromolar range. Unexpectedly, the most potent inhibitors found are those that possess a trimethylammonium group but have chemically blocked the zinc coordinating functionalities. The results obtained suggest that PC-PLC$_{Bc}$ inhibition is not due to the interaction of compounds with the phospholipase catalytic zinc atoms, but rather results from the inhibitor cationic group recognition by the PC-PLC$_{Bc}$ amino acids involved in choline lipid binding.

1. Introduction

Phospholipases C (PLC) constitute a class of lipid phosphohydrolase enzymes that are involved in the hydrolysis of the phosphodiester bond in phospholipids to provide a lipidic alcohol and a phosphorylated polar head group. Phospholipases play a key role in different biological processes, such as cell membrane homoeostases,[1] digestion,[2,3] inflammation,[4–6] infection[7,8] and signal transduction.[9–11] In mammals the products released by the action of phosphatidylcholine-specific phospholipase C (PC-PLC), diacylglycerol and phosphocholine, are involved in cell function and signalling.[12–16] Just to cite a few examples, PC-PLC is involved in hepatocarcinogenesis,[17] ovarian tumour progression[18] or leukaemia.[19] In contrast to phosphatidylinositol phospholipases C,[20] mammalian PC-PLCs are poorly characterised, and no eukaryotic PC-PLC has thus far been isolated and purified. The discovery that *Bacillus cereus* phosphatidylcholine-specific phospholipase C (PC-PLC$_{Bc}$) had an antigenic similarity with mammalian PC-PLC[21] prompted the use of this well known bacterial PLC, as a model for its mammalian counterpart. Therefore, the discovery of inhibitors of PC-PLC$_{Bc}$ opens up a way to the development of mammalian PC-PLC inhibitors and their use as pharmacological tools for the study of this enzyme.

PC-PLC$_{Bc}$ is a small, monomeric enzyme, with three Zn^{2+} atoms in the active site likely to be involved in the binding to the substrate[22–24] and essential for the enzymatic activity and protein conformational stability.[25] Chemical modifications of a specific carboxyl group or histidine, lysine and arginine residues result in enzyme inactivation,[26–30] this indicating they are essential residues for catalytic activity. Comparison of the structures of PC-PLC$_{Bc}$ variants with that of the wild-type enzyme suggests that minor changes in steric and electronic properties in the binding site of PC-PLC$_{Bc}$ are responsible for significant changes in substrate selectivity.[31]

To date, several inhibitors of PC-PLC$_{Bc}$ have been identified. Among them, D609, occupies a prominent place. This potent compound inhibits the activity of PC-PLC$_{Bc}$ while not affecting the activities of phospholipase D and phosphatidylinositol-specific

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phospholipase C. The synthesis in our group of all four diastereomers of D609 and the lack of significant differences in their inhibitory activity suggests an absence of diastereochemical control of the enzyme by xanthate inhibitors.[32] Besides its PC-PLC$_{Bc}$ inhibitory activity, D609 has been reported as inhibitor of other enzymes such as sphingomyelin synthase, acidic sphingomyelinase and a group IV cytosolic phospholipase A_2.[33] As a consequence, the effects of this inhibitor on PC-PLC$_{Bc}$ are far from being selective. Moreover, D609 is a potent antioxidant[34] and is able to modulate sphingolipid transport.[35] With this plethora of options, the identification of cellular targets involved in D609 activities remains obscure. Zinc-chelating molecules like univalent anions,[36] Tris buffer[23] or cyclic *N,N′*-hydroxyureas[37] have been found to inhibit PC-PLC$_{Bc}$ as well. Their interaction with the three metal ions in the protein molecule[22,23,36] prevents enzymatic activity, a fact that confirms the importance of the metal ions in the catalytic mechanism. Phosphate and phosphocholine analogues constitute another group of PC-PLC$_{Bc}$ inhibitors; all of them are compounds able to prevent the catalytic activity by interaction with the active site of the enzyme.[30,38–40]

The strategy followed by our group to design PC-PLC$_{Bc}$ inhibitors was based on the structural similarities between PC-PLC$_{Bc}$ and other zinc enzymes.[41] The comparison of the structures *Streptomyces griseus* and *Aeromonas proteolytica* aminopeptidases with that of PC-PLC$_{Bc}$ showed a notable structural similarity, especially in the relative disposition of catalytic zinc atoms involved in the hydrolytic reactions promoted by these enzymes. Starting from known inhibitors of the aforementioned aminopeptidases, we designed and synthesized a small series of α-aminohydroxamic acids and α-aminophosphonic acids and studied their activity as phospholipase inhibitors, to know if the similarities in the tridimensional active sites of the enzymes could also be extended to these inhibitors. This approach was successful, and α-aminohydroxamic acids, initially reported as aminopeptidase inhibitors, were found to be also potent PC-PLC$_{Bc}$ inhibitors.[41]

In this article we describe the modifications introduced in the 2-aminohydroxamic scaffold to improve the inhibitor properties on PC-PLC$_{Bc}$. The X-ray analysis of the complex of PC-PLC$_{Bc}$ and a phosphonate inhibitor showed that the trimethylammonium group, corresponding to the phosphonate polar head, interacts with certain active site amino acid residues that are important for an effective binding with the protein.[23] With the aim of achieving a stronger and more selective inhibitor–enzyme interaction, a choline-like group has been added to the hydroxyamide nitrogen of the above α-aminohydroxamic acids. Since α-aminohydroxamic PC-PLC$_{Bc}$ inhibition was found to be non-enantioselective,[41] we decided to employ racemic amino acids in this study. Further analysis of the binding model proposed by Martin et al.[30] for the enzyme–substrate interaction suggested that the binding of the inhibitor to the enzyme active site might be modulated by the length of the alkyl tether chain between the trimethylammonium group and the hydroxyamide nitrogen of the polar head (Fig. 1). So, two series of analogues, containing two or three methylene units, were obtained (Fig. 1) and tested as inhibitors of PC-PLC$_{Bc}$.

2. Results

2.1. Synthesis of compounds

The target molecules were obtained coupling an *N*-Boc protected *n*-alkyl-2-amino acid with a N-alkylated hydroxylamine. Amino acid **1** was obtained by *N*-Boc protection of commercial 2-aminooctanoic acid, whereas longer alkyl amino acids **2**–**4** were synthesized following classical procedures described in the literature,[42–44] followed by amine protection by treatment with di-*tert*-butyldicarbonate to give intermediates **5**–**7** (Scheme 1).

The N-substituted hydroxylamines to be coupled with the amino acids were obtained following the synthetic pathway depicted in Scheme 2. *O*-Benzylhydroxylamine was reacted with di-*tert*-butyldicarbonate in alkaline medium to give the *N*-Boc protected derivative **8**. Treatment of compound **8** with NaH, followed by reaction of the resulting anion with 1,2-dichloroethane or 1,2-dichloropropane resulted in nitrogen alkylation to give carbamates **9a** and **9b**. The next step was the S_N2 substitution of the terminal chlorine with dimethylamine as nucleophile in a reaction that furnished diamines **10a** and **10b**. Deprotection of compounds **10a** and **10b** under acidic conditions gave the target *O*-benzyl protected hydroxylamines **11a** and **11b**.

Compounds **11a** and **11b** were reacted with the required amino acids using EDC/HOBt standard coupling conditions in moderate yields. For compounds with a short alkyl tether chain in the choline group, the tertiary amine was converted into a quaternary ammonium salt by treatment with MeI. Subsequent deprotection of the hydroxyl and amino groups gave α-aminohydroxamic acids **24**–**27** (Scheme 3).

The synthesis of the second group of analogues, those with a longer alkyl tether chain in the choline group, was carried out as described in Scheme 4. Only 2-aminooctanoic acid and to 2-aminododecanoic acid derivatives were prepared in this series, since biological studies performed on compounds **16**–**27** showed a clear correlation between the alkyl chain length and cytotoxicity in mammalian cells (Table 3). Moreover, to determine the importance of the presence of a quaternary ammonium group for the inhibitory activity, two different classes of compounds were synthesized, one having a dimethylamino substituent in the polar head (compounds **28**–**33**) and the other having a trimethylammonium group (compounds **34**–**39**). The synthetic approach, depicted in Scheme 4, was similar to the above described, involving the coupling of *N*-Boc amino acids **1** and **5** to diamine **11b**, to give intermediates **28** and **29**, followed by amine methylation, when appropriate, and final *N*-Boc and *O*-benzyl deprotection steps.

Whereas the removal of *N*-Boc groups under acidic conditions in the 2-aminohydroxamic derivatives was successful, the *O*-benzyl deprotection proved problematic and required considerable experimentation. For compounds having a dimethylamino substituent, the hydrogenolysis worked nicely with Pd/C catalyst at room temperature and 1 atm H_2. In contrast, debenzylation of compounds having quaternary trimethylammonium groups under the above conditions failed. We attempted first to deprotect the *N*-Boc derivatives, but the hydrogenolysis to obtain the *N*-Boc 2-aminohydroxamic acids failed in all cases with different palladium catalysts (Pd/C, $Pd(OH)_2$/C, $Pd/BaSO_4$) obtained from different commercial sources and different batches. Several reaction temperatures (from rt to 80 °C) and hydrogen pressure (1–3 atm) and different reaction times (up to 5 days) were attempted. In general, no reaction was observed at low temperature and/or pressure conditions. When forcing hydrogenation conditions we observed mixtures of the starting material, accompanied by variable amounts of the desired *N*-hydroxyamide and the amide resulting from reduction of the N–O bond. These types of compounds predominate when reaction times were extended to complete the consumption of the *O*-benzyl starting materials. This side reaction is described in literature[45], in a related hydrogenolysis of a *O*-benzyl hydroxamate in cyclic 3-amino-1-hydroxypyrrolidin-2-one systems. In this kind of molecules a fine tuning of the reactivity of the substrate and catalyst is required for successful hydrogenolysis of the benzyl group without overreduction of the hydroxyamide to amide. These precedents prompted us to try the debenzylation of the corresponding free α-ammonium compounds arising from previous *N*-Boc removal.

This strategy proved effective and compounds **20** and **21** (Scheme 3) were debenzylated with 10% $Pd/BaSO_4$ catalyst

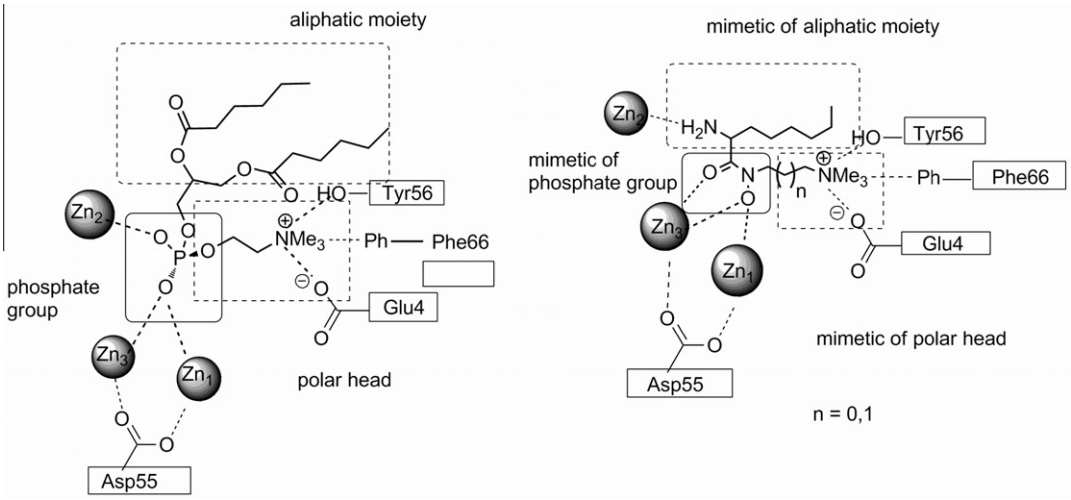
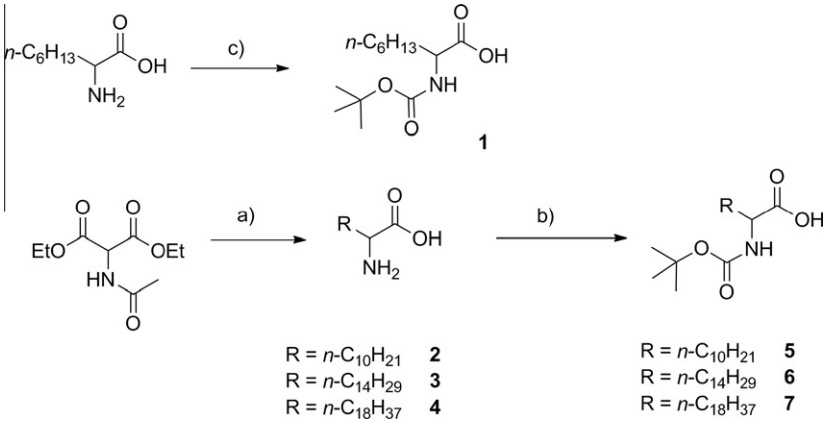
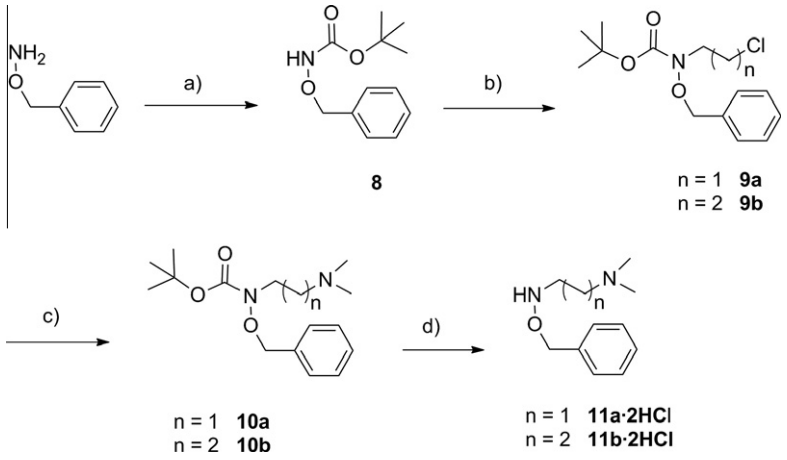


Figure 1. Binding model for enzyme–substrate interaction [adapted from Martin and co-worker[52]] and for enzyme–2-aminohydroxamic inhibitor interaction.

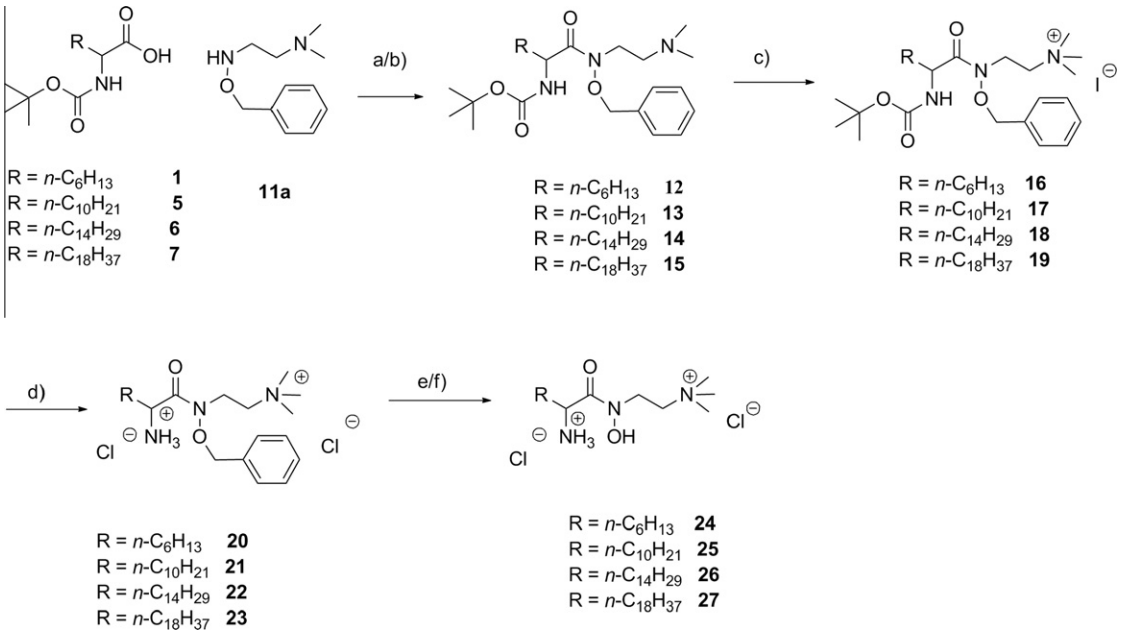


Scheme 1. 2-Amino acid synthesis. Reagents, conditions and yields: (a) $(^tBuOCO)_2O$, TEA/CH_2Cl_2, 80%; (b) $Br(CH_2)_9CH_3$ (for **2**), $Br(CH_2)_{13}CH_3$ (for **3**), $Br(CH_2)_{17}CH_3$ (for **4**) NaOEt/EtOH, reflux; then HCl, reflux; (c) $(^tBuOCO)_2O$, NaOH/dioxane (59% for **5**, 49% for **6**, 33% for **7**).

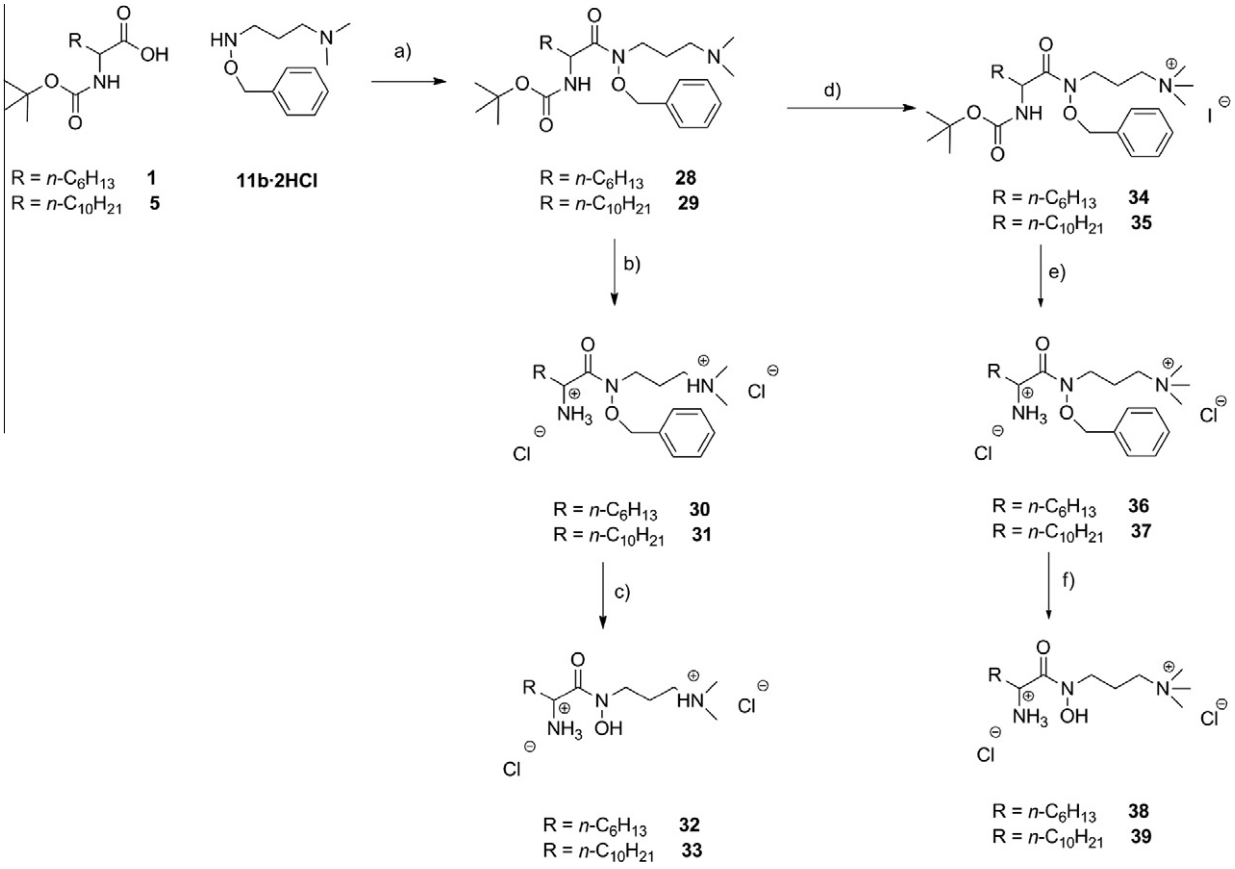


Scheme 2. Synthesis of compounds **11a** and **11b**. Reagents, conditions and yields: (a) $NaHCO_3$, $(^tBuOCO)_2O$, H_2O/CH_2Cl_2; (b) *n* = 1: 1,2-dichloroethane, NaH/DMF, 77% global yield; *n* = 2: 1,3-dichloropropane, NaH/DMF, 70%; (c) dimethylamine, NaI, EtOH, 80 °C, *n* = 1: 78%; *n* = 2: 74%; (d) HCl, MeOH, *n* = 1: 99%; *n* = 2: 100%.

(3 atm H_2, room temperature, 24 h) to give excellent yields of the corresponding hydroxamic acids **24** and **25**. However, for the longer alkyl derivatives **22** and **23** this catalyst was not operative and the more active Pearlman's catalyst was required to obtain the corresponding hydroxamic acids **26** and **27** in acceptable to good yields. For the series having three methylene units in the polar head, the *O*-benzyl derivatives were deprotected in a very similar way (Scheme 4). As in the shorter derivatives, the reactivity of



Scheme 3. Synthesis of compounds **20**–**23**. Reagents, conditions and yields: (a) EDC/HOBt, TEA/CH_2Cl_2, 60 °C, 60 h; (b) EDC/HOBt, TEA/THF, 60 °C, 60 h (43% for **12**, 31% for **13**, 30% for **14**, 31% for **15**); (c) MeI, CH_2Cl_2, 24 h, rt (99% for **16**, 99% for **17**, 99% for **18**, 100% for **19**); (d) HCl, MeOH, 2 h, 0 °C (99% for **20**, 99% for **21**, 99% for **22**, 100% for **23**); (e) 10% Pd/$BaSO_4$, 3 atm H_2, MeOH, 24 h, rt (99% for **24**, 98% for **25**); (f) 20% Pd(OH)$_2$/C, 3 atm H_2, MeOH, 24 h, rt (55% for **26**, 88% for **27**).



Scheme 4. Synthesis of compounds **28**–**39**. Reagents, conditions and yields: (a) EDC, HOBt, TEA/THF, 60 h, 60 °C [45% for **28**, 56% for **29**]; (b) HCl, MeOH, 2 h, 0 °C (96% for **30**, 99% for **31**); (c) H_2, Pd/C, 1 atm, 24 h (99% for **32**, 88% for **33**); (d) MeI, CH_2Cl_2, 18 h, rt (99% for **34**, 99% for **35**); (e) HCl, MeOH, 2 h, 0 °C (99% for **36**, 99% for **37**); (f) H_2, Pd(OH)$_2$, 3 atm, 24 h (40% for **38**, 52% for **39**).

the dimethylamino derivatives was higher than that of the trimethylammonium compounds. Thus, **30** and **31** were converted into hydroxamic acids **32** and **33** with Pd/C and 1 atm of H_2 in excellent yields. Trimethylammonium compounds **36** and **37** were

unreactive under these conditions but could be deprotected under 3 atm of H_2 using $Pd(OH)_2/C$ as catalyst, to give **38** and **39** in 40% and 52% yields, respectively. In contrast with the related compounds with two methylene units, the use of $Pd/BaSO_4$ as catalyst for O-debenzylation in **36** and **37** was ineffective.

2.2. Inhibition of PC-PLC$_{Bc}$

The PC-PLC$_{Bc}$ activity of final products and intermediates was measured according to the method described by Hergenrother and Martin[46] which is based on the quantification of phosphate ions arising from alkaline phosphatase (AP) catalysed hydrolysis of phosphorylcholine produced by PC-PLC$_{Bc}$. Given that AP is a Zn-phosphoesterase and to avoid possible interferences in the assay, we studied the effect of the tested compounds on AP, concluding that its activity was not affected at 0.5 mM (data not shown).

Since PC-PLC$_{Bc}$ exhibits greatly increased activity on a micellar environment,[47] the assay is best conducted below the critical micelle concentration (CMC) of amphiphilic molecules present. The substrate used in this case was 1,2-di-*n*-hexanoyl-*sn*-glycero-3-phosphocholine, at 2 mM final concentration, well below its CMC of 11.1 mM. We also calculated CMC values for the α-aminohydroxamic acid derivatives, to ensure that the enzymatic assay was performed under non-micellar conditions. As expected, CMC values decreased as long chain lipophilicity increased (Tables 1 and 2). As a general trend, for the same R substituent, CMC values increased as protective groups were removed and dimethylammonium derivatives exhibited higher CMC than the corresponding trimethylammonium analogues.

As shown in Table 1, the compounds obtained from 2-aminooctanoic acid were weak inhibitors of PC-PLC$_{Bc}$, since three out of the six compounds resulted to be inactive and the rest exhibited IC_{50}'s around 50–100 μM. The inhibitory activities found for the remainder members of the trimethylammonium derivative families ranged the low micromolar IC_{50}'s and exhibited two remarkable trends. Inhibitory activity increased roughly with the alkyl chain length and, for a given alkylamino acid derivative, the compounds having *N*-Boc and *O*-benzyl protecting groups were among the more active ones. This unexpected result implies that the zinc coordinating functionalities are not required for inhibition. Similar results were obtained for compounds with a three methylene bridge ($n = 2$) and a dimethylamino in the polar head. A higher inhibitory activity was observed for compounds with a longer alkyl chain (Table 2). Comparison of the results depicted in Tables 1 and 2 show very similar activities for inhibitors with a two ($n = 1$) and three ($n = 2$) methylene bridges.

We previously had reported that α-aminohydroxamic acids competitively inhibited PC-PLC$_{Bc}$.[41] To know about the inhibition modes of the new inhibitors, some K_i values were determined. Compound **25**, with a trimethylammonium group and a two methylene bridge, compounds **35** and **39**, with a trimethylammonium group and a three methylene bridge and compound **29**, with a dimethyl amino group and a three methylene bridge were chosen.

The results obtained showed that there was not a common pattern of inhibition. Thus, compound **25** (K_i, 12 μM) appeared to be an uncompetitive inhibitor whereas compounds **35** (K_i, 2.5 μM) and **39** (K_i, 26 μM) showed a mixed type inhibition and compound **29** (K_i, 2.5 μM) was a non-competitive inhibitor. Analysis of the results obtained indicates that, for aminohydroxamic compounds **25** and **29** that have a trimethylammonium group, the introduction of an extra methylene between the trimethylammonium group and the hydroxyamide nitrogen caused a change in the inhibition type. Whereas **25** was an uncompetitive inhibitor, compound **39** at low substrate concentrations was also uncompetitive, while at high substrate concentrations they behaves as a competitive inhibitor. A similar mixed inhibition mode was obtained for compound **35**, which has the amino and hydroxamate functionalities blocked. The related compound **29** which posses a dimethylamino group was found to be a PC-PLC$_{Bc}$ non-competitive inhibitor.

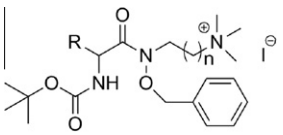
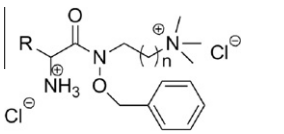
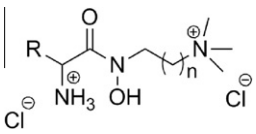
To rule out that PC-PLC$_{Bc}$ inhibition was originated from protein denaturalisation by the inhibitors, compound **18** and octadecyl-2-aminohydroxamic acid, one of our previously described PC-PLC$_{Bc}$ inhibitor lacking the choline-like group,[41] were independently incubated in the presence of PC-PLC$_{Bc}$ at a concentration in which the enzymatic inhibition was around 80% of the untreated enzyme. After 10 min, the medium was diluted to reach an inactive inhibitor concentration and the enzymatic activity was determined. In both cases the enzymatic activity was around 100% (data not shown). This result clearly indicates that these compounds do not affect irreversibly the enzyme activity, excluding an unspecific inhibition due to protein denaturation by the inhibitors.

Next, we checked the effect of the compounds on cell growth using the MTT test. Regarding trimethylammonium derivatives (Table 3), none of the compounds with a 6-carbon alkyl chain was found out to be toxic. Longer alkyl chains were more toxic and their toxicity decreased as the protective groups were removed (see LD_{50} values for **18**, **22** and **26**, for example). Dimethylamino compounds (Table 4) exhibited lower LD_{50} values than the corresponding trimethylammonium analogues.

3. Discussion

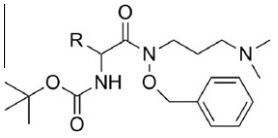
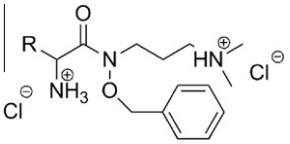
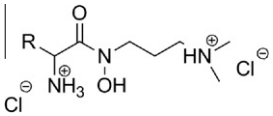
Our initial objective was to improve the PC-PLC$_{Bc}$ inhibitory activity of a family of new α-aminohydroxamic PC-PLC$_{Bc}$ inhibitors we had previously disclosed[41] by adding to these structures a choline like group. These inhibitors were found among compounds

Table 1
Inhibition (IC_{50} values, μM) of PC-PLC$_{Bc}$ by 2-aminohydroxamic acid derivatives

R	*n*									
		Compd	CMC	IC_{50}	Compd	CMC	IC_{50}	Compd	CMC	IC_{50}
n-C_6H_{13}	1	**16**	–[a]	56	**20**	–[a]	–[b]	**24**	–[a]	500
	2	**34**	221	78	**36**	210	100	**38**	–[a]	–[b]
n-$C_{10}H_{21}$	1	**17**	–[a]	5	**21**	–[a]	10	**25**	–[a]	40
	2	**35**	40	4	**37**	141	50	**39**	97	58
n-$C_{14}H_{29}$	1	**18**	–[a]	2	**22**	90	4	**26**	120	2
n-$C_{18}H_{37}$	1	**19**	75	3	**23**	10	4	**27**	50	4

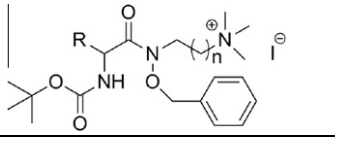
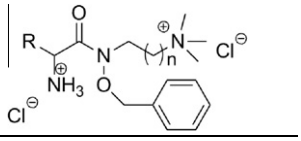
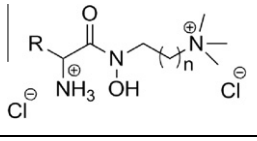
[a] CMC value higher than 500 μM.
[b] IC_{50} value higher than 500 μM.

Table 2
Inhibition (IC_{50} values, μM) of PC-PLC$_{Bc}$ by 2-aminohydroxamic acid derivatives with a dimethylamino group

R									
	Compd	CMC	IC_{50}	Compd	CMC	IC_{50}	Compd	CMC	IC_{50}
C_6H_{13}	**28**	75	77	**30**	158	—[b]	**32**	—[a]	—[b]
$C_{10}H_{21}$	**29**	10	8	**31**	8	32	**33**	79	100

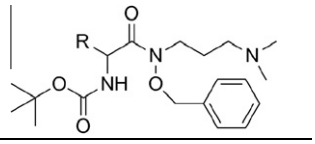
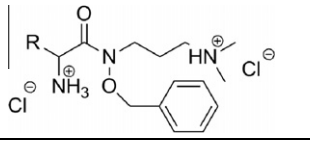
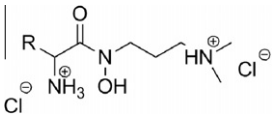
[a] CMC value higher than 500 μM.
[b] IC_{50} value higher than 500 μM.

Table 3
Cytotoxicity of 2-aminohydroxamic acid derivatives

R									
	n	Compd	LD_{50}	*n*	Compd	LD_{50}	*n*	Compd	LD_{50}
n-C_6H_{13}	1	**16**	—[a]	1	**20**	—[a]	1	**24**	—[a]
	2	**34**	—[a]	2	**36**	—[a]	2	**38**	—[a]
n-$C_{10}H_{21}$	1	**17**	28.2	1	**21**	76.1	1	**25**	—[a]
	2	**35**	17.0	2	**37**	43.4	2	**39**	—[a]
n-$C_{14}H_{29}$	1	**18**	27.6	1	**22**	75.3	1	**26**	100
n-$C_{18}H_{37}$	1	**19**	25.1	1	**23**	29.0	1	**27**	52.7

[a] LD_{50} value higher than 100 μM.

Table 4
Cytotoxicity of 2-aminohydroxamic acid derivatives

R						
	Compd	LD_{50}	Compd	LD_{50}	Compd	LD_{50}
C_6H_{13}	**28**	21	**30**	—[a]	**32**	—[a]
$C_{10}H_{21}$	**29**	6	**31**	7	**33**	40

[a] LD_{50} value higher than 100 μM.

that inhibited enzymes with active sites having structural similarity with PC-PLC$_{Bc}$. Inhibitor modifications, derived from the analysis of the complex of PC-PLC$_{Bc}$ and a phosphonate inhibitor,[48] and a binding model of the hydroxamic compounds led us to introduce alkylamine substituents in the hydroxyamide nitrogen to mimic the choline present in the phosphatidylcholine PC-PLC$_{Bc}$ substrate. The series obtained contained an ethyltrimethylammonium group and resulted in compounds **16**–**27**. Study of their activity on PC-PLC$_{Bc}$ showed that they were potent inhibitors with IC_{50}'s in the low micromolar range, similar to the α-aminohydroxamic inhibitors. The importance of the interaction between the trimethylammonium group and certain residues of the enzyme, led us to further modify the structure of the inhibitors by an elongation of the polar head alkyl tether chain. This second modification produced compounds **28**–**39**, another series having several inhibitors with IC_{50} in the low micromolar range and some inactive compounds.

The results obtained gave us some insights about the structural features that favour inhibition of PC-PLC$_{Bc}$. From our initial work on xanthate[32] and α-aminohydroxamic[41] PC-PLC$_{Bc}$ inhibitors we concluded that the presence of a zinc binding group and a lipophilic chain were essential for potent inhibitory activity. However, we found that final compounds having the zinc coordinating functionalities (the amino group and/or the *N*-hydroxyl group), such as **24**, **25**, **32**, **33** and **38**, were less active than their *N*-Boc and/or *O*-benzyl synthetic precursors, while others, like compounds **26**, **27** and **39**, had activities similar but not higher than those of protected synthetic intermediates. Rather unexpectedly, the inhibitor zinc coordination, initially thought to be essential for the inhibitory activity, can be avoided provided that a charged trimethylammonium (or even dimethylammonium) group is present in the lipophilic chain presumably to mimic the choline group of PC-PLC$_{Bc}$ substrates. To confirm this, we tested hexadecyltrimethylammonium chloride (cetrimide) in the phospholipase assay and we confirmed that this was certainly a potent PC-PLC$_{Bc}$ inhibitor with a IC_{50} of 4 μM. However, cetrimide toxicity (LD_{50} 1 μM) precludes its use in cellular studies of PC-PLC$_{Bc}$ inhibition. This result confirms the importance of the charged ammonium salt for enzyme inhibition in the compounds studied, in accordance with the important role played by choline in the recognition of phospholipid substrates by the enzyme.[31] As for the importance of hydrophobic interactions, this had already been reported[49,50] and we had observed a

similar trend in xanthate PC-PLC$_{Bc}$ inhibitors,[32] where the binding of the compounds to the active site depends on the presence of the zinc coordinating dithiocarbonate group, with the present hydrophobic moiety modulating its inhibitory activity.

The increase of the alkyl tether length from two to three carbon atoms did not result in a substantially higher inhibitory potency. Compounds from the first and second series exhibited similar IC_{50} values. Overall, it seems that inhibitory activity increases with the lipophilicity of the molecule that can be the result of either a longer amino acidic alkyl aminohydroxamic functionalities. The compounds having dimethylamino groups in place of trimethylammonium substituents display inhibitory activities in the same order of magnitude but systematically less potent than their trimethylammonium counterparts. Four compounds, representative of the different inhibitor types studied, were selected for kinetic studies and we found that none of them was a pure PC-PLC$_{Bc}$ competitive inhibitor. Compound **25** was an uncompetitive inhibitor, compound **29** turned out to inhibit the enzyme in a non-competitive way and compounds **35** and **39** were mixed inhibitors. The lack of interaction of the compounds with the active site of the enzyme would explain why the zinc coordinating moiety is not essential for inhibitory activity. This behaviour is different from that of other PC-PLC$_{Bc}$ competitive inhibitors synthesized in our group, such as alpha-aminohydroxamic acids or xanthathes, where a low inhibitory potency was found when the zinc coordinating groups were chemically blocked. Although these results cannot be generalised to all members of the different chemical families studied, we think that they are compatible with inhibitor binding to PC-PLC$_{Bc}$ protein sites other than the zinc catalytic centre, a fact that might be related to the known interfacial activation of this enzyme.[51] The cytotoxicity exhibited for long chain analogues and the low inhibitory activity elicited by *n*-hexylamino acid derivatives, points out to *n*-decanoyl derivatives as the best candidates to develop future work on in vivo studies of this new family of PC-PLC inhibitors.

4. Experimental

Detailed experimental procedures and spectral data for the synthesized compounds are provided in Supplementary data.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.10.031.

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