D-Tyrosine as a Chiral Precusor to Potent Inhibitors of Human Nonpancreatic Secretory Phospholipase A_2 (IIa) with Antiinflammatory Activity

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Few reported inhibitors of secretory phospholipase A_2 enzymes truly inhibit the IIa human isoform (hnpsPLA$_2$-IIa) noncovalently at submicromolar concentrations. Herein, the simple chiral precursor D-tyrosine was derivatised to give a series of potent new inhibitors of hnpsPLA$_2$-IIa. A 2.2-Å crystal structure shows an inhibitor bound in the active site of the enzyme, chelated to a Ca^{2+} ion through carboxylate and amide oxygen atoms, H-bonded through an amide NH group to His48, with multiple hydrophobic contacts and a T-shaped aromatic-group – His6 interaction. Antiinflammatory activity is also demonstrated for two compounds administered orally to rats.

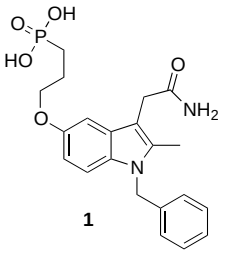
KEYWORDS:

enzymes · inflammation · inhibitors · medicinal chemistry · structure – activity relationships

Introduction

The phospholipase A_2 (PLA_2) class of enzymes[1] catalyse hydrolysis of the 2-acyl ester of 3-*sn*-phosphoglycerides[1e] to yield arachidonic acid (metabolised to eicosanoids by cyclooxygenase and lipoxygenase) and lysophospholipid (converted into platelet activating factor).[2] The enzymes tend to hydrolyse substrate aggregates like monolayers, micelles, vesicles and membranes.[3] Mammalian tissues contain both secretory ($sPLA_2$ groups I, IIA, IIC, V, X) and cytosolic ($cPLA_2$ group IV) enzymes that require Ca^{2+} ions for activity,[2] as well as a Ca^{2+}-independent intracellular isoform (group VIA $iPLA_2$).[4] Although relationships between isoforms, relative capacities to degrade membrane phospholipids and specific isoform physiology remain uncertain, there is substantial evidence in support of pathogenic roles for PLA_2.

For example, $sPLA_2$ and $cPLA_2$ enzymes have been directly implicated in eicosanoid production[5–7] and human nonpancreatic secretory PLA_2 isoform IIa (hnpsPLA$_2$-IIa) has been found to be secreted from many eicosanoid-producing cell types,[2c, 8] which include human platelets,[9] neutrophils[10] and mast cells.[11] HnpsPLA$_2$-IIa has also been found in abnormally high concentrations in human synovial fluid from rheumatoid[12] and osteoarthritis patients and in blood from patients with burns, sepsis, asthma, pancreatitis, psoriasis, Crohn's disease, adult respiratory distress syndrome and atherosclerosis.[1a–d, 2] There is a strong correlation between severity of disease and $sPLA_2$ levels, and intravenous administration of hnpsPLA$_2$-IIa to rabbits also produces symptoms of arthritis and sepsis.[13] Most reported hnpsPLA$_2$-IIa inhibitors lack potency[14] but some show efficacy[14a, 15] in animal models of inflammation, and an analogue of **1**[16] is in clinical trials. We now report: 1) potent inhibitors **2a**–**q** of hnpsPLA$_2$-IIa derived from D-tyrosine, 2) a crystal structure for **2b** complexed with the enzyme, and 3) preliminary anti-inflammatory activity in rats.



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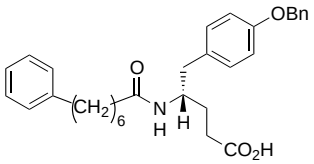

2b

Synthesis

We used glycerophospholipid substrates as a basis to construct chiral substrate analogues (Scheme 1, 2), derived simply by using

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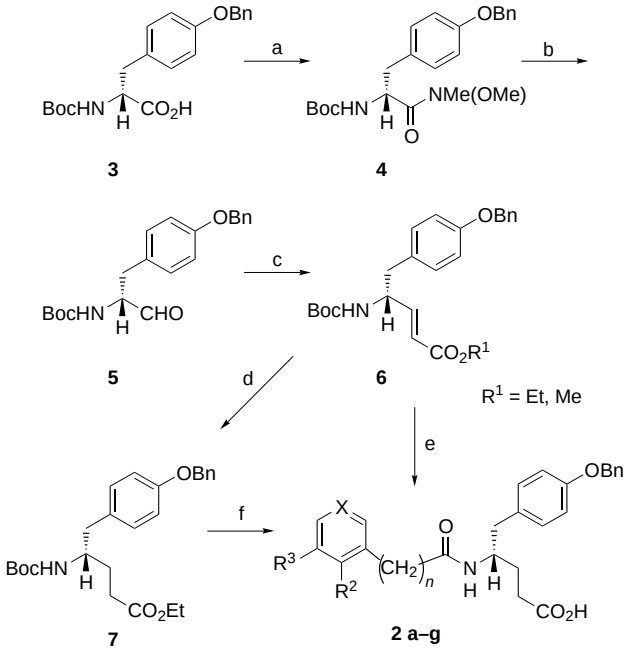
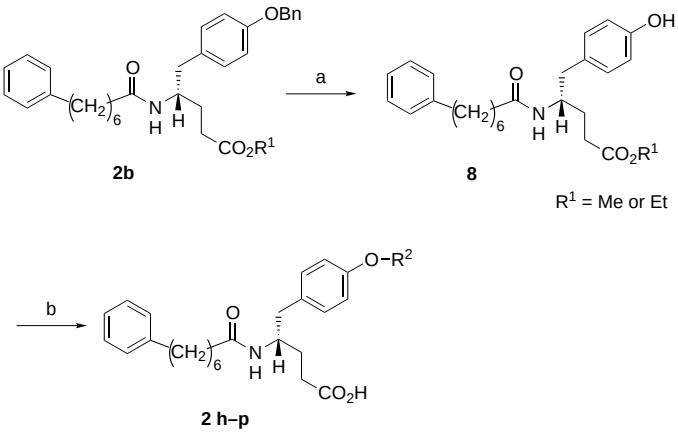


Scheme 1. Reagents: a) BOP, DIPEA, DMF, NHMe(OMe)·HCl; b) $LiAlH_4$, THF; c) $Ph_3P{=}CHCO_2Me$ or $Ph_3P{=}CHCO_2Et$, THF; d) Pd/C, H_2, EtOAc; e) (i) TFA, CH_2Cl_2, (ii) BOP, DIPEA, DMF, substituted or unsubstituted phenyl- or pyridylalkanoic acid, (iii) H_2, Pd/C, EtOAc, (iv) aq. NaOH, THF, MeOH, (v) 1 M HCl; f) (i) TFA, CH_2Cl_2, (ii) BOP, DIPEA, DMF, 7-(3-nitro-phenyl)-heptanoic acid, (iii) aq. NaOH, THF, MeOH (iv) 1 m HCl. Boc = *tert*-butoxycarbonyl, Bn = benzyl, BOP = benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate, DIPEA = N,N-diisopropylethylamine, DMF = dimethylformamide, THF = tetrahydrofuran, Ac = acetyl, TFA = trifluoroacetic acid.



Scheme 2. Reagents: a) H_2, Pd/C, THF, HCl; b) (i) R^2–X (X = Cl, Br, I), K_2CO_3, DMF, (ii) NaOH, THF, MeOH.

the commercially available Boc-D-tyrosine (Boc = tert-butoxcarbonyl) derivative **3** as a chiral template for the series of compounds **2a**–**q**, which inhibit hnpsPLA$_2$-IIa in vitro.

Inhibitors **2a**–**g** (Table 1) were prepared from benzyl-protected Boc-D-tyrosine **3** according to the synthesis shown in Scheme 1, which involves conversion to the Weinreb[17a] amide **4**, reduction with $LiAlH_4$ to aldehyde **5** and homologation with either methyl- or ethyl(triphenylphosphoranylidene)acetate to give intermediate **6**. Mosher analysis[17b] of the amine corresponding to **6** confirmed that *S* stereochemistry was preserved. Compounds **2a**–**f** were synthesised directly from **6** by sequential deprotection (TFA in CH_2Cl_2), coupling of the appropriate pyridyl or phenyl alkanoic acid derivative[17c] and hydrogenation over Pd/C in ethyl acetate (Scheme 1). Saponification with NaOH produced the inhibitor series **2a**–**f**.

Inhibitor **2g** was prepared by hydrogenation of **6** over Pd/C in ethyl acetate to give **7**. The benzyl ether was stable under these conditions. Removal of the Boc group with TFA, BOP-mediated coupling of the liberated amine with 7-(3-nitro-phenyl)-heptanoic acid[17c] and saponification gave **2g**. Inhibitors **2h**–**p** (Table 2) were prepared from the ester of **2b** (R^1 = Me, Et) as shown in Scheme 2. The benzyl group was removed by hydrogenolysis over Pd/C in THF/HCl to give **8**, which was alkylated with the appropriate alkyl halide in the presence of K_2CO_3, and the ester was hydrolysed under alkaline conditions to give **2h**–**p**.

Table 1. *Compounds from Scheme 1 and their relative potencies as inhibitors of the enzyme hnpsPLA$_2$-IIa.*[a]

Structure	R^2	R^3	X	n	IC_{50} [μM][b]	Mole fraction (Xi)
1	-	-	-	-	0.060	0.000045[16]
2a	H	H	CH	5	0.662	0.00044
2b	H	H	CH	6	0.029	0.000019
2c	H	H	CH	7	2.48	0.0017
2d	OMe	H	CH	6	1.82	0.0012
2e	H	NHAc	CH	6	4.05	0.0027
2f	H	H	N	6	0.761	0.00051
2g	H	NO_2	CH	6	0.536	0.00036

[a] Determined in a chromogenic assay[18] by using Ellman's reagent to detect cleavage of a thioester substrate. [b] IC_{50} = the concentration of inhibitor required to achieve 50% inhibition.

Table 2. *Compounds from Scheme 2 and their relative potencies as inhibitors of the enzyme hnpsPLA$_2$-IIa.*[a]

Structure	R^2	IC_{50} [μM]	Mole fraction (Xi)
2h	2-picolyl	0.214	0.00014
2i	3-picolyl	0.247	0.00017
2j	cyclohexylmethyl-	0.067	0.000045
2k	cyclopentylmethyl-	0.057	0.000038
2l	1-napthylmethyl-	0.019	0.000013
2m	2-napthylmethyl-	0.039	0.000026
2n	cinnamyl-	0.116	0.000078
2o	iso-butyl	0.170	0.00011
2p	n-heptyl	0.086	0.000058
2q	H	2.57	0.0017

[a] Determined in a chromogenic assay[18] by using Ellman's reagent to detect cleavage of a thioester substrate.

Enzyme Inhibition

Compounds **2a**–**q** were evaluated as inhibitors by using an *in vitro* colorimetric enzyme assay,[18] which showed that inhibition correlates strongly with *in vivo* antiinflammatory activity in rats (for example, against arthritis, ischemia-reperfusion injury). Thirteen of the seventeen compounds **2a**–**q** have submicromolar inhibitory potencies against hnpsPLA$_2$-IIa (Tables 1, 2)

under conditions in which indole **1** has comparable *in vitro* potency (Table 1). Like **1**, compounds **2a**–**q** are also potent *in vitro* inhibitors of rat uterine contractions and of rat models of arthritis and rat intestinal ischemia-reperfusion injury (manuscripts containing these results are in preparation).

Crystal Structure

During the past decade, X-ray crystal structures have been reported for human $cPLA_2$[19] and $sPLA_2$-IIa[20–24] bound to different substrate-derived inhibitors ranging from transition state and substrate analogues[12, 20–25] to indoles,[16] indolizines[24] and carboxamides,[25] and there is now also a structure[26] for the enzyme $sPLA_2$-X. The information provided by these structures will guide the design and development of new and possibly isoform-selective inhibitors that can be used to probe the significance of each of these enzymes in disease.

Compound **2b** was cocrystallised with $hnpsPLA_2$-IIa by the sitting drop method (20 °C, 0.1 M tris(hydroxymethyl)aminomethane (Tris), pH 7.4) under previously reported conditions.[23] The X-ray crystal structure (2.2-Å resolution, space group P31, $a = b = 75.11$ Å, $c = 50.03$ Å, $\alpha = \beta = 90°$, $\gamma = 120°$) shows **2b** bound within the hydrophobic active site cavity[27] very much like other known inhibitors[20–25] (Figure 1). In fact, the key enzyme-binding components (two calcium-binding oxygen atoms, a His48-binding H-bond donor, and a hydrophobic component) that bind in the active site of this enzyme are virtually superimposable for all known inhibitors. Thus, the amide carbonyl oxygen atom and one carboxylate oxygen atom are chelated to the Ca^{2+} ion, with enzyme residues Asp49, His48, Gly30 and Gly32 completing the octahedral 6-coordinate environment of calcium. The amide NH group of **2b** makes an important[28] hydrogen bond with the catalytic residue His48, analogous to the transition state for substrate hydrolysis.

All of the inhibitors possess hydrophobic chains that mimic the sn-1 and sn-2 aliphatic chains[1e] of endogenous substrates and have similar interactions with enzyme residues. The phenylheptanoyl chain corresponding to the sn-2 position of the endogenous substrate (Figure 1) lies deep within the active site cavity and makes numerous close contacts (< 4.3 Å) with the enzyme through the hydrophobic residues Phe5, Ile9, Ala18, Ala19, Tyr22, Gly23 and Cys45, which line the cavity. The phenyl ring displaces the imidazole ring of His6, a residue unique to human $sPLA_2$-IIa, and forms edge-to-face T-shaped interactions with His6 and the tyrosinyl phenyl ring of the sn-1[1e] chain. The O-benzyl group of **2b** has been modelled in two alternative conformations,[27] each of which allow it to make additional close contacts with hydrophobic enzyme residues Leu2, Phe24 and either Ala18 or Val31.

Structure – Activity Relationships

An extended conformation for the phenylheptanoyl chain of **2b** (Figure 1) appears to be required for optimal filling of the hydrophobic active site of the enzyme, since alteration of the length of this chain by subtraction (**2a**, $IC_{50} = 0.6$ μM) or addition (**2c**, $IC_{50} = 2.5$ μM) of just one methylene unit reduces inhibitory potency by up to 100-fold (Table 1). This optimal length (six

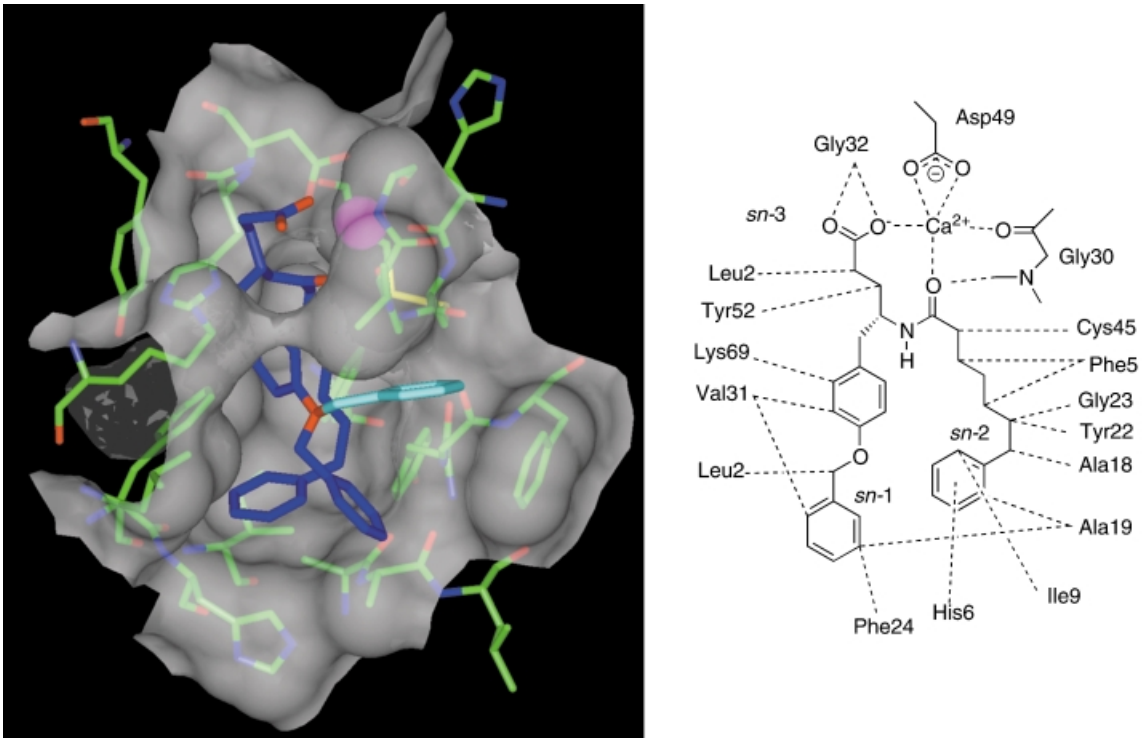


Figure 1. Left: Crystal structure of **2b** (dark blue) in the active site of $hnpsPLA_2$-IIa (grey), with nearby enzyme residues (green), Ca^{2+} (pink) and an alternative orientation[27] of O-benzyl (light blue). Right: Representation of other interactions between enzyme residues and **2b**.

methylene groups) for **2b** has similarly been reported for another inhibitor series.[23, 29] His6, which is displaced by the terminal phenyl ring of the phenylheptanoyl chain, lies at the base of the hydrophobic cavity and is unique[12] to the human enzyme. It is similarly displaced upon binding of other substrate analogues[12, 23] to hnpsPLA$_2$(IIa). The terminal phenyl ring also forms a nonbonded intramolecular interaction with the tyrosine-derived aromatic ring, the two rings being perpendicular to one another. Such non-bonded interactions may contribute significantly toward the binding affinity of **2** to hnps-PLA$_2$, although there is controversy about the importance of such T-shaped edge-to-face interactions in small molecules and proteins.[30]

In an attempt to modulate putative edge-to-face interactions between His6 and the terminal phenyl ring derived from the phenylheptanoyl chain, we incorporated electron-donating (*o*-methoxy, **2d**; *m*-acetamido, **2e**) and electron-withdrawing (*m*-nitro, **2g**) substituents into the phenyl ring (Table 1), but found reduced inhibitory potency in each case. Substitution of the terminal phenyl ring for a pyridine ring also resulted in loss of activity (**2f**, $IC_{50} = 0.76\ \mu M$). These results suggest that polar functional groups are not well tolerated within the hydrophobic cavity, and that the edge-to-face interactions are either unaffected by electronic changes or do not contribute much to inhibitor binding.[30]

To improve the solubility of **2b** (Log $D_{7.5} = 4.06$, calculated with the Pallas 2.1 program, CompuDrug Chemistry Ltd., Hungary), we replaced the benzyloxy group with a pyridinemethoxy group, with moderate loss of activity (Table 2: **2h**, $IC_{50} = 0.21\ \mu M$; **2i**, $IC_{50} = 0.25\ \mu M$), which suggests a preference for nonpolar groups in the hydrophobic region defined by residues Leu2, Ile9, Ala19, Phe24 and Val31 (Figure 1). Removal of the benzyloxy group altogether (**2q**) dramatically reduces activity, but replacement of the benzyloxy group by various alkyl, cycloalkyl or naphthyl hydrophobic substituents (**2j**–**2p**) alters potency within only a fivefold range.

Efficacy In Vivo

We conducted a very preliminary investigation (Table 3) of the efficacy of two of the above compounds (**2b**, **2h**) in a standard systemic rat model of chronic adjuvant-induced arthritis[31] that has been used extensively to evaluate numerous antiinflammatory drugs. Injection of complete Freund's adjuvant at the tail base of a rat results in a polyarthritis that usually manifests itself after 12 days as inflammation of the tail and all four paws, and lesions on the forepaws and ears. The arthritic syndrome, measurable on Day 14 by paw swelling, resembles to some extent human arthritic conditions such as rheumatoid arthritis. Herein, we use this assay as a simple gauge of the likely *in vivo* efficacy of compound series **2** given orally to rats in a single dose ($5\ mg\ kg^{-1}\ day^{-1}$ on Days 10–13). Results on Day 14 (Table 3) show substantial inhibition of the oedema following oral administration of these compounds on Days 10–13 inclusive. In addition, histology of the spleens from drug-treated rats showed mild capsular oedema and minimal infiltration of macrophages into the red pulp, which indicates that both compounds were effective in preventing the histological damage and splenomegaly seen for untreated arthritic controls. Overall, the results suggest that further investigation of this compound series as antiinflammatory agents may be warranted.

The expression of sPLA$_2$ and cPLA$_2$ enzymes has been monitored during the course of this adjuvant arthritis model in Lewis rats.[32] A maximal increase of sPLA$_2$ mRNA was observed in paws, lung and aorta (32 μmol/30′) on Day 14, and in lymph nodes and spleen on Day 28, with negligible protein expression in the liver, whereas cPLA$_2$ mRNA levels remained unchanged during the course of the disease. The observed parallel between expression of sPLA$_2$ and severity of disease is consistent with sPLA$_2$ having a pathogenic role, and supports the anti-inflammatory activity observed in this model for hnpsPLA$_2$-IIa inhibitors **2b** and **2h**.

There remains considerable uncertainty about the relative merits of sPLA$_2$ (for example, IIa, V, X) versus cPLA$_2$ enzymes as prospective targets for anti-inflammatory drugs. However, the reports that indole analogues of **1** have anti-inflammatory activity *in vivo*,[15] together with the findings that oral administration of the structurally quite different compounds described here (such as **2b**, **2h**) also show antiinflammatory activity in vivo in a systemic model of chronic inflammatory disease, and a recent report[33a] that **2b** also inhibits myocardial ischemia in Lewis rats, lend support to the idea that inhibitors of sPLA$_2$ do have value as antiinflammatory drugs.

Conclusion

A strategy in which D-amino acids are used as templates for the development of sPLA$_2$ inhibitors[33b] is exemplified herein by the simple synthesis of 17 compounds **2a**–**q** from the chiral precursor D-tyrosine. Thirteen of the compounds had submicromolar inhibitory potency against hnpsPLA$_2$-IIa *in vitro*. Compound **2b** was co-crystallised with hnpsPLA$_2$-IIa and the crystal structure showed **2b** bound within the active site in the expected binding mode. Two of the compounds (**2b**, **2h**) were

Table 3. *Inhibition of adjuvant arthritis (Day 14) in Wistar rats by oral administration (Day 10–13) of **2b** and **2h**.*

Treatment[a]	n[b]	Rear paw swelling (cm)[c]	Forepaw inflammation (score)[d]	ΔWt (g)[e]
Vehicle only	12	0.80 ± 0.06	$2.9 + (\pm 0.3)$	+02
2b ($5\ mg\ kg^{-1}\ day^{-1}$)	8	0.04 ± 0.03	$0.2 + (\pm 0.2)$	+04
2h ($5\ mg\ kg^{-1}\ day^{-1}$)	8	0.18 ± 0.03	$0.7 + (\pm 0.2)$	+01

[a] Administered orally on Days 10–13 after inoculation with arthritogen (*Mycobacterium tuberculosis* in squalane) on Day 0. [b] Number of rats. [c] Mean ± standard error of the mean, measured by microscrew gauge. [d] Average subjective score (0–4+) for severity of lesions and swelling. [e] Weight change over Days 10–13.

orally administered to rats and showed antiinflammatory activity *in vivo*. The results support the notion that $sPLA_2$-IIa may indeed be important in the pathogenesis of inflammatory disease, although this work does not discount the possibility that other isoforms of $sPLA_2$ are similarly inhibited *in vivo*. Clearly more extensive testing of inhibitors of this enzyme against other forms of $sPLA_2$ and in other animal models of inflammatory disease is warranted.

Supporting Information: The crystal structure coordinates have been deposited in the Protein Data Bank (code 1j1a). Synthetic procedures and characterisation data for compounds **2a–q**, details of the enzyme assay, crystal structure and crystallographic information files (37 pages) are available free of charge as supporting information.

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[18] Each of 96 wells contained a total solution volume of 225 μL, which consisted of enzyme (110 ng hnpsPLA_2-IIa), substrate (1.66 mM 1,2-bis(heptanoylthio)-1,2-dideoxy-sn-glycero-3-phosphorylcholine), 0.3 mM Triton X-100 mixed micelles, buffer (25 mM Tris–HCl buffer, pH 7.5, 10 mM $CaCl_2$, 100 mM KCl, 1 mg mL^{-1} bovine serum albumin), 5,5′-dithionitrobenzoic acid (Ellman's reagent), ± test inhibitor in dimethyl sulfoxide (5 μL). Mixtures were incubated at 37 °C, and enzyme activity was measured by reaction of cleaved thioester substrate with Ellman's reagent, quantified by absorbance at 414 nm. This assay is described in the Cayman Chemicals assay kit handbook (http://www.caymanchem.-com/index.html) and in: L. J. Reynolds, L. L. Hughes, E. A. Dennis, *Anal. Biochem.* **1992**, *204*, 190–197.

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