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Parallel synthesis and *in vitro* activity of novel anthranilic hydroxamate-based inhibitors of the prostaglandin H_2 synthase peroxidase activity†

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Currently available non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin are directed at the cyclooxygenase (COX) site, but not the peroxidase (POX) activity of prostaglandin H_2 synthase (PGHS). They are thus unable to inhibit the free-radical induced tissue injury associated with PGHS peroxidase activity, which can occur independently of the COX site. A lead compound, anthranilic hydroxamic acid (AHA) was found to have significant PGHS-POX inhibitory activity ($IC_{50} = 72$ μM). To define the critical parameters for PGHS-POX inhibition, we investigated 29 AHA derivatives, synthesised from their acid precursors, using solid phase synthesis. *In vitro* analysis demonstrated a ten-fold improvement in inhibition with 3,5-diiodoanthranilic hydroxamic acid ($IC_{50} = 7$ μM).

Introduction

Prostaglandin H_2 synthase (PGHS), or cyclooxygenase (COX), is the enzyme catalysing the first two steps in the biotransformation of arachidonic acid to the prostaglandin hormones.[1–5] There are different isozymes of PGHS encoded by distinct genes: PGHS-1, the constitutive enzyme expressed in most tissues and PGHS-2, which can be described in a simplified manner as the inducible isoform, expressed at sites of inflammation and in tumours. A third isozyme derived from the PGHS-1 gene has recently been identified.[6]

PGHS is a bifunctional enzyme possessing two connected active sites,[7] a cyclooxygenase site (COX), which catalyses the addition of two oxygen molecules to arachidonic acid and a spatially and functionally distinct peroxidase site (POX), which catalyses the reduction of a hydroperoxide group to the corresponding alcohol. The COX active site is the target of non-steroidal anti-inflammatory drugs (NSAIDs), such as aspirin.[8] The peroxidase activity is unaffected by NSAIDs and can function independently of COX site inhibition.[9,10] Compounds targeting the POX site are of potential therapeutic value, as unchecked free radical generation contributing to disease progression in inflammatory conditions could occur despite NSAID administration.[11] Also, nitric oxide, which induces vasodilation and inhibits platelet aggregation, is consumed during PGHS-POX activity.[12] Furthermore, as cyclooxygenase catalysis is dependent on POX activity,[1,13] inhibition of the peroxidase site would also lead to inactivation of the COX site. Therefore, there is a two-fold reason for developing PGHS peroxidase site inhibitors. Compared to the number of available COX inhibitors, a relatively small number of POX inhibitors have been described. These include synthetic *N*-acyl-hydroxylamines and -hydrazines,[14] as well as natural peroxidase inhibitors such as resveratrol, the cardio-protective, anti-inflammatory and cancer preventive compound found in red wine.[15]

We have previously reported that acetylated salicylhydroxamate derivatives can act as effective inhibitors of the COX activity of PGHS by acetylating a serine residue in this active site.[16,17] Triacetylsalicylhydroxamic acid in particular can potentially acetylate two molecules of PGHS, *via* the formation of an intermediate acetylating species, *O*-acetylsalicylhydroxamic acid, following first-step acetylation. These reactions would finally yield one molecule of salicylhydroxamic acid (SHA, **2**, Fig. 1), a known inhibitor of myeloperoxidase.[18] As numerous peroxidases are inhibited by aromatic hydroxamic acids such as benzohydroxamic acid (BHA, **1**),[19–21] we investigated if SHA, a potential metabolite of our COX inhibitors, could in addition interfere with the POX activity of PGHS. SHA was not an effective inhibitor of the H_2O_2-dependent oxidation of a POX co-substrate by PGHS. We thus sought more potent peroxidase inhibitors, based on the aromatic hydroxamic acid backbone. Such molecules substituted on the *ortho* position with a group capable of forming hydrogen bonds represent the minimal pharmacophore for the generation of COX and POX inhibitors.[14] We therefore turned our attention to anthranilic hydroxamic acid (AHA, **3**, Fig. 1) which we found inhibited peroxidase activity of PGHS with an IC_{50} value of 72 μM (*vide infra*) and was deemed a suitable lead compound for optimisation by parallel solid phase synthesis.

We report here the synthesis of targeted combinatorial arrays of anthranilic hydroxamic acids and their *in vitro* activity as potential inhibitors of the peroxidase catalysis of PGHS.

† Electronic supplementary information (ESI) available: schemes of 2-chlorotrityl chloride polystyrene resin and *p*-nitrophenyl carbonate Wang resin based methods; table of conditions for modified Wang resin method; correlation between biological activity and electronic properties of substituted anthranilic hydroxamic acids (-AHA) MOPAC calculations; QSAR analysis; plots of PGHS-1 peroxidase inhibition *vs.* hydrophobicity and molecular density; NMR spectra. See http://dx.doi.org/10.1039/b505525c

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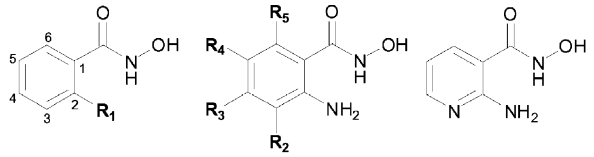


(1-3) (3-30) (31)

		R_1
1	BHA	H
2	SHA	OH
3	AHA	NH_2

		R_2	R_3	R_4	R_5
3	AHA	H	H	H	H
4	3-chloro AHA	Cl	H	H	H
5	3-methoxy AHA	OCH_3	H	H	H
6	3-trifluoromethyl AHA	CF_3	H	H	H
7	4-chloro AHA	H	Cl	H	H
8	4-fluoro AHA	H	F	H	H
9	4-nitro AHA	H	NO_2	H	H
10	5-acetamido AHA	H	H	$NHCOCH_3$	H
11	5-bromo AHA	H	H	Br	H
12	5-chloro AHA	H	H	Cl	H
13	5-fluoro AHA	H	H	F	H
14	5-hydroxy AHA	H	H	OH	H
15	5-iodo AHA	H	H	I	H
16	5-methoxy AHA	H	H	OCH_3	H
17	5-methyl AHA	H	H	CH_3	H
18	5-nitro AHA	H	H	NO_2	H
19	6-chloro AHA	H	H	H	Cl
20	6-fluoro AHA	H	H	H	F
21	6-methyl AHA	H	H	H	CH_3
22	3,5-dichloro AHA	Cl	H	Cl	H
23	3,5-dibromo AHA	Br	H	Br	H
24	3,5-diiodo AHA	I	H	I	H
25	3,5-dimethyl AHA	CH_3	H	CH_3	H
26	3-bromo-5-methyl AHA	Br	H	CH_3	H
27	3,4,5-trimethoxy AHA	OCH_3	OCH_3	OCH_3	H
28	3,4,5,6-tetrafluoro AHA	F	F	F	F
29	4,5-difluoro AHA	H	F	F	H
30	4,5-dimethoxy AHA	H	OCH_3	OCH_3	H
31	Nicotinic AHA				

Fig. 1 Anthranilic hydroxamic acid (AHA) derivatives synthesised.

The synthetic protocol used is an extension of methodology previously reported by us in this journal for monofunctional hydroxamic acids.[22] Of the 29 anthranilic hydroxamic acids synthesised, 22 are reported for the first time.

Results and discussion

The effect of substitution on the aromatic ring of AHA on peroxidase activity inhibition has been evaluated in this study. A library of 29 anthranilic hydroxamic acid derivatives containing a wide range of substituents with different electronic and steric properties have been prepared and tested (see Fig. 1).

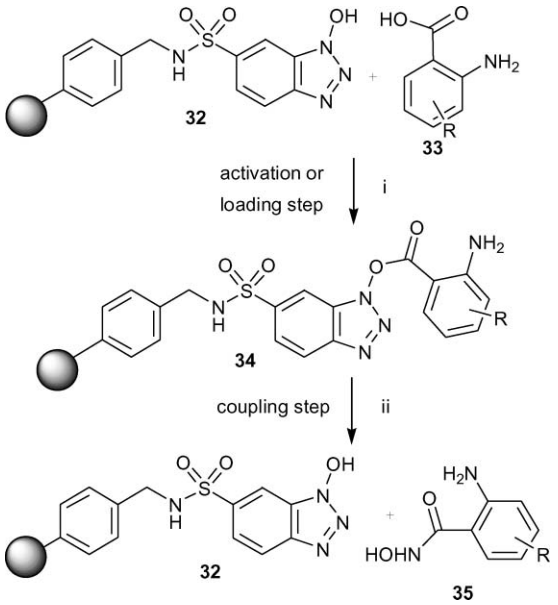
Solid phase synthesis of anthranilic hydroxamic acids

The general strategy was to prepare the novel AHA compounds by solid phase synthesis, from commercially available carboxylic acid precursors, without the need to protect the anilino group. An attractive solution is to use this latter function for the attachment of anthranilic acid to the resin, and to subsequently convert the free carboxylic group to the hydroxamate by simple coupling chemistry with hydroxylamine.

The first resin selected was the 2-chlorotrityl chloride polystyrene resin, which allows the anchoring of compounds through a nucleophilic group and their later release under mild acidic conditions (ESI,† Fig. S1). The loading of the resin with anthranilic acid (4 equivalents relative to resin) was attempted in the presence of diisopropylethylamine (2 equivalents relative to resin) to generate the free anilino group. However, FTIR analysis of the modified resin with anthranilic acid showed that the attachment of this substrate occurred through either the anilino or the carboxylic group (data not shown). Therefore, the reduced nucleophilicity of this amino group does not afford unequivocal loading of the trityl chloride resin.

A *p*-nitrophenyl carbonate Wang resin has previously been used for the successive immobilisation of anthranilic acid through its anilino group, amidification of its carboxylic group and cyclization-cleavage to generate quinazoline-2,4-diones.[23] We followed the reported procedure[23] for the modification of the Wang resin and the loading of anthranilic acid by formation of a carbamate linkage (ESI,† Fig. S2). The success of this latter step was verified by sacrificing a small portion of the resin to directly liberate anthranilic acid under acidic conditions. The polymer-bound anthranilic acid was then used for the acylation of hydroxylamine. However, despite several attempts, based on different coupling chemistries (carbodiimides, phosphonium and uronium reagents, a preformed active *p*-nitrophenyl ester prepared according to Mitsunobu conditions, preformed mixed anhydrides) and conditions using protected or free hydroxylamine and on different cleavage conditions, poor yields and purities for the anthranilic hydroxamic acid, not exceeding 50% and 42% respectively, were obtained (see ESI,† Table S1).

The reduced nucleophilicity of the amino group in anthranilic acid evidenced previously (*vide supra*) prompted us to assess the preparation of the hydroxamates by nucleophilic displacement of resin-bound anthranilate active esters with hydroxylamine. This 'catch-and-release' strategy is based on the formation of a polymer-bound active ester in a first step and its reaction with hydroxylamine in a second step. It was therefore hoped that the anilino group would not displace the intermediate resin-bound anthranilate to form the 2-(2-amino-benzoylamino)benzoic acid. We selected two polystyrene-based supports, the oxime resin, a well established support for the solid phase synthesis of hydroxamic acids[24] and the hydroxybenzotriazole (HOBt) resin **32** (Scheme 1), which was recently used by our group for the preparation of low molecular weight hydroxamic acids.[22] In our hands, the best results were obtained with the HOBt-active esters **34**. The reactivity of these intermediates afforded shorter reaction times, for both the activation and the coupling steps (2 h and 4 h respectively for the HOBt esters *versus* 17 h and 18 h respectively for the oxime esters) and a lower number of equivalents of hydroxylamine than for their oxime esters counterparts (0.9 equivalents for the HOBt esters *versus* 3 equivalents for the oxime esters). Moreover, the hydroxamic acids **35** were obtained in quantitative yields and in high purities



Scheme 1 'Catch-and-release' method. *Reagents and conditions*: i, PS-HOBt (1 equiv.), anthranilic acid (1.5 equiv.), diisopropylcarbodiimide, (4.5 equiv.), 4-(dimethylamino)pyridine, (0.6 equiv.), 1 : 1 DCM–DMF, 2 h; ii, hydroxylamine (0.9 equiv.), 2.3 : 1 THF–MeOH, 4 h.

(see Experimental section). Finally, the recycling of the resin **32** was successfully undertaken to scale-up the preparation of some hydroxamic acids to gram quantities (data not shown). The by-products, detected by reverse-phase HPLC, were the carboxylic acids **33**, accounting for 2%, in the case of compound **10**, to 23%, in the case of compound **28**, of the isolated products. Traces of 4-(dimethylamino)pyridine or *N*,*N*′-diisopropylurea were also observed by HPLC or NMR, respectively, in some cases. We did not investigate if 2-(2′-amino-benzoylamino)benzoic acid derivatives were formed during the activation step, as the final yields of hydroxamic acids were satisfactory. In addition, this unwanted product would have been eliminated during the washes performed after this step. In the case of *N*,*N*′-diisopropylurea we prepared an authentic sample and tested it in the TMPD assay (*vide infra*). Finally, some hydroxamic acids from the library (**17**, **20**, **22**, **24** and **26**) covering a range of biological activities were selected and purified by reverse-phase HPLC to a satisfactory analytical purity. The biological activities of these purified hydroxamic acids were assessed and found to be equivalent to those of the non-purified compounds (see Table 1, footnote *c*).

In vitro biological activity

TMPD assays. The compounds were screened for their ability to inhibit the PGHS-1 and -2 peroxidase activity by measuring the H_2O_2-dependent oxidation of the chromogenic reducing co-substrate, *N*,*N*,*N*′,*N*′-tetra-*p*-methylphenylenediamine (TMPD)[25] (Table 1, Fig. 2). Inhibitors, at a range of concentrations, were pre-incubated with the enzyme before both the co-substrate (TMPD) and substrate (H_2O_2) were added to start the reaction. Initial rates of reaction were measured.

All carboxylic acid precursors of these compounds showed no inhibition of either isozyme in this assay (*i.e.* IC_{50} > 1000 μM),

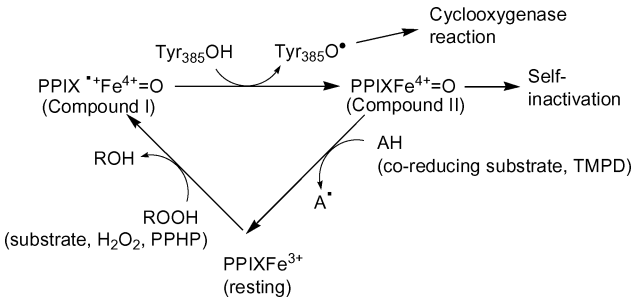


Fig. 2 The PGHS-peroxidase reaction.

indicating that the hydroxamic moiety is necessary for peroxidase inhibition. This is consistent with previous observations on tepoxalin and naproxen hydroxamic acid, both of which are PGHS-1 peroxidase inhibitors, whereas their carboxylic acid derivatives are inactive.[25] Both the 4-(dimethylamino)pyridine and diisopropylurea were also shown to be inactive (IC_{50} > 1000 μM in both cases). Therefore, the inhibitory activity observed is solely due to the hydroxamic acids.

Our initial observation was that the relative potency for the inhibition of PGHS-1 peroxidase activity was AHA > BHA > SHA (**1–3**, Table 1). To examine the effect of the relative position of the amino group in AHA, we tested 3-amino BHA and 4-amino BHA[26] and found IC_{50} values of 146 ± 14 μM and 373 ± 47 μM respectively, both significantly higher than AHA which has an IC_{50} value of 72 ± 26 μM. The preference shown by the PGHS-1 peroxidase site for an amino group at the *ortho* position led us to investigate the influence of substitution at different positions on the phenyl ring of anthranilic hydroxamic acid (**4–31**).

Of the 29 compounds synthesised, 3,5-diiodoanthranilic hydroxamic acid (3,5-diiodo AHA, **24**) was the most effective

Table 1 Inhibition of PGHS peroxidase activity by substituted anthranilic hydroxamic acids (-AHA)

-AHA	IC_{50} TMPD PGHS-1[a]	IC_{50} TMPD PGHS-2[a]	% PPHP[b] PGHS-1
3,5-I_2 (**24**)	7.2 ± 3.1[c]	16 ± 10	16 ± 11
3,5-Br_2 (**23**)	12.2 ± 7.1	132 ± 92	34 ± 8
3,5-Cl_2 (**22**)	12.9 ± 8.3[c]	148 ± 90	58 ± 3
5-Br (**11**)	13.0 ± 2.3	128 ± 55	49 ± 12
3-Cl (**4**)	14 ± 12	577 ± 178	79 ± 13
4,5-F_2 (**29**)	15 ± 7	108 ± 30	69 ± 18
5-I (**15**)	17 ± 13	134 ± 34	52 ± 16
3-Br-5-Me (**26**)	23 ± 5[c]	257 ± 142	77 ± 2
4-Cl (**7**)	27 ± 10	184 ± 250	64 ± 0
5-NO_2 (**18**)	28 ± 18	315 ± 98	79 ± 4
5-Cl (**12**)	29 ± 18	284 ± 73	75 ± 3
4-F (**8**)	35 ± 9	498 ± 156	78 ± 14
3,4,5,6-F_4 (**28**)	43 ± 8	184 ± 49	80 ± 0
3-CF_3 (**6**)	49 ± 59	241 ± 45	78 ± 20
5-F (**13**)	68 ± 91	357 ± 132	65 ± 20
4-NO_2 (**9**)	69 ± 18	191 ± 97	59 ± 20
AHA (**3**)	72 ± 26	425 ± 199	76 ± 13
3,5-$(CH_3)_2$ (**25**)	84 ± 12	402 ± 123	83 ± 0
Nicotinic (**31**)	84 ± 86	>1000	63 ± 6
5-OH (**14**)	110 ± 41	220 ± 153	70 ± 3
5-CH_3 (**17**)	115 ± 55[c]	263 ± 88	67 ± 8
BHA (**1**)	116 ± 12	>1000	n.d.
3-OCH_3 (**5**)	116 ± 82	>1000	87 ± 22
4,5-$(OCH_3)_2$ (**30**)	206 ± 91	109 ± 43	83 ± 0
5-$NHCOCH_3$ (**10**)	248 ± 83	>1000	78 ± 3
SHA (**2**)	378 ± 50	>1000	n.d.
5-OCH_3 (**16**)	388 ± 235	304 ± 216	85 ± 1
6-F (**20**)	419 ± 264[c]	>1000	65 ± 7
6-Cl (**19**)	>1000	>1000	62 ± 3
6-CH_3 (**21**)	>1000	>1000	60 ± 12
$(OCH_3)_3$ (**27**)	>1000	>1000	76 ± 19

[a] IC_{50} values for inhibition of TMPD oxidation shown in μM (200 μM H_2O_2 substrate; 200 μM TMPD co-substrate; $n = 3$, ± std. dev.). [b] Percentage of maximal PPHP conversion using 200 μM of each compound as co-reducing agent ($n = 2$ to 4, ± std. dev.). [c] IC_{50}s in μM of purified AHAs are: 5.3 ± 1.8 for **24**; 8.4 ± 0.8 for **22**; 30.3 ± 18.3 for **26**; 93.0 ± 18.3 for **17**; 921.6 ± 66.1 for **20**.

inhibitor, with IC_{50} values of 7.2 ± 3.1 μM and 16 ± 10 μM for PGHS-1 and PGHS-2 respectively (Table 1). This was closely followed by the other dihalogenated compounds 3,5-dibromo AHA (**23**), 3,5-dichloro AHA (**22**) and 4,5-difluoro AHA (**29**) with IC_{50} values of 12.2 ± 7.1 μM, 12.9 ± 8.3 μM and 15 ± 7 μM, respectively. Monohalogenated compounds such as 5-bromo AHA (**11**), 3-chloro AHA (**4**), and 5-iodo AHA (**15**) were also found to be effective, with IC_{50} values of 13.0 ± 2.3 μM, 14 ± 12 μM and 17 ± 13 μM, respectively for inhibition of PGHS-1 peroxidase activity.

Whereas most of the compounds showed inhibitory activity towards the peroxidase site of PGHS-1, apart from 3,5-diiodo AHA, all were ineffective as inhibitors towards the peroxidase site of PGHS-2, with IC_{50} values greater than 100 μM. This is not surprising since the peroxidase catalyses of PGHS-1 and -2 are markedly different.[5] However, our results allow for selective inhibition of the peroxidase site of PGHS-1.

PPHP assays. A limitation frequently overlooked in peroxidase inhibitor studies is that the assay is based on the oxidation of a chromogenic reducing co-substrate (*e.g.* TMPD or guaiacol) and not on the reduction of the substrate (*e.g.* H_2O_2) itself. Therefore, apparent inhibitors may compete with TMPD oxidation, rather than exhibit true inhibition of enzyme catalysis. Consequently the IC_{50} values reflect a competition with the co-reducing assay reagent (TMPD), and not the substrate itself (H_2O_2). We have addressed this in our studies by assaying the reduction of a lipid peroxide substrate (*trans*-5-phenyl-pent-4-en-1-yl hydroperoxide, PPHP) to the corresponding alcohol (*trans*-5-phenyl-pent-4-en-1-ol, PPA), using HPLC[27] (Table 1). At 200 μM, most of the AHA derivatives showed significant reducing co-substrate activity, exceeding that of phenol (set at approximately 40% in this assay). However, compounds **23** and **24**, showed less co-reducing activity than phenol. Compound **24** (3,5-diiodo AHA) showed no activity as a reducing co-substrate at 200 μM (*i.e.* well in excess of the TMPD IC_{50}), indicating that it is likely a true inhibitor of the PGHS-1 peroxidase site.

The effects of substituents on biological activity. Aromatic hydroxamic acids have been proposed to act as reducing co-substrates in the peroxidase catalytic cycle of PGHS.[14] Reaction with compound II (Fig. 2) would presumably yield an oxygen-centered free radical that could be delocalised by resonance through the phenyl ring *via* a hydroxamate tautomer.[28] A radical of this type would be expected to be stabilised in *ortho* and *para* positions, while the influence of *meta* substituents should be slightly destabilising or negligible.[29–31]

From Table 1, it is obvious that compounds with substituents *meta* to the hydroxamate, and thus *ortho* or *para* to the amino group, are the best inhibitors. Electron-withdrawing groups (-Cl, -Br, -F, and $-CF_3$) as well as -I increase potency. In addition to their inductive effect, -Br and -Cl can stabilize a free radical by resonance.[29–31] Significantly, this is also true for the more polarisable -I. 3,5-Dihalogenated derivatives are the most active, but the difference between their activities and those of the monohalogenated analogues is very small. For instance, comparing **4** (3-chloro AHA) with **22** (3,5-dichloro AHA), and **11** (5-bromo AHA) with **23** (3,5-dibromo AHA) indicates that even one halogen substituent at positions 3 or particularly 5 (*meta* to the hydroxamate and *ortho* or *para* to the amino group, respectively) produces an inhibitor nearly as active as the dihalogenated compounds. The low activity of compound **10** (5-acetamido AHA) may be due to the steric demands of the acetamido substituent outweighing its electron withdrawing properties.

Electron-donating non-polarisable groups ($-CH_3$, -OH, and $-OCH_3$), on the other hand, reduce inhibition, all compounds containing such groups (**25**, **31**, **14**, **5**, **17**, **30**, **16**, **21** and **27**) showing less activity than AHA. Interestingly, the only exception, 3-bromo-5-methyl AHA (**26**), which is more effective than AHA, has the halogen substitution *meta* to the hydroxamate, showing that for a *meta* substituent, an electron-withdrawing group overrides the effect of an electron-releasing methyl group.

Assuming a free radical mechanism, the effects of substituents *ortho* or *para* (positions 3 and 5) to the amino group are more consistent with the formation of a nitrogen-centered aniline radical[32] as opposed to an oxygen centered hydroxamate radical. This is despite the fact that semiempirical calculations show that the enthalpy of formation of the latter is significantly lower (see QSAR studies below). In any case, it appears that the amino and hydroxamate groups must be in *ortho* positions for activity to be observed.

An alternative explanation is that the substituents in position 5 establish favourable interactions with the active site, thus helping the compound to adopt a different binding conformation. Different binding modes may account for the differing levels of inhibition of the compounds: if the free radical generated upon reaction with compound II diffuses away, the molecule would act as a mere reducing co-substrate, whereas if the free radical reacts with a residue within the active site resulting in protein damage the molecule would be an inhibitor.

The fact that only **24** (3,5-diiodo AHA) seems to be a genuine inhibitor of PGHS-1, and possibly PGHS-2, may suggest a different mechanism of action for this particular compound. A two-electron oxidation of compound **24** to yield an iodine(III) species[33] could result in enzyme inactivation.

Another significant finding is that substituents at the 6 position (**19**, **20** and **21**), *ortho* to the hydroxamic acid, render the compound ineffective as an inhibitor in the TMPD assay. This could be explained because of the steric hindrance in the environment of the reactive centre (assuming that it is the hydroxamate). However, *ortho* substituents would also force the carbonyl group out of plane with respect to the phenyl ring, thus destabilizing a free radical centered on the hydroxamate oxygen, and the amino nitrogen.

QSAR and modelling studies. Semiempirical calculations were carried out for all the compounds using MOPAC at PM3[34] level of theory (results are given in ESI†). As already observed in similar QSAR studies[35] the correlation found between the computed electronic properties of the compounds described herein and their IC_{50} values was poor (see ESI†). Among the properties analyzed were the heats of formation of three possible free radicals (hydroxamate oxygen, hydroxamate nitrogen and aniline nitrogen), ionization potentials and electron densities and bond orders at the likely reactive centres of the inhibitors (ESI,† Table S2).

Docking calculations were also carried out with a few representative compounds using the program Autodock[36] and a crystal structure[37] of the ovine PGHS-1 (Protein Data Bank accession number 1Q4G) and a compound II model prepared with SYBYL (Tripos Inc.). The docking energies obtained are very similar for all the compounds (approximately −8.0 kcal mol^{-1}), about one half of the value found for the natural substrate PGG_2 (data not shown, manuscript in preparation).[38] We conclude that the enzyme–inhibitor interactions are weak and essentially non-specific.

A QSAR analysis was undertaken using the QuaSAR package of the MOE[39] suite of programs, using 203 2D and 3D descriptors, as reported previously.[40] A weak correlation was found between the hydrophobicity coefficient [log*P*(o/w), octanol/water] and the pIC_{50} for PGHS-1 peroxidase inhibition [$pIC_{50} = -\log(IC_{50} \times 10^{-6})$, where IC_{50} is in μM] (ESI,† Fig. S3). This is reasonable as the effective concentration within an essentially hydrophobic cavity is expected to be higher for the less water-soluble compounds.

Conclusion

In contrast to other peroxidases, PGHS-1 appears to favour an amino group at the *ortho* position in the aromatic hydroxamic acid peroxidase inhibitors, *i.e.* anthranilic hydroxamic acid.

Derivatisation showed that substituents *ortho* and *para* to the amino group, *i.e. meta* to the hydroxamate, are preferred, with electron-withdrawing or polarisable substituents significantly improving inhibition. Electron-donating groups, however, consistently decrease efficacy. All aromatic hydroxamic acid peroxidase inhibitors tested showed significant co-substrate activity, except for 3,5-diiodoanthanilic hydroxamic acid, which appears to be a true PGHS-1 peroxidase inhibitor.

Experimental

Materials

The 1-hydroxybenzotriazole-6-sulfonamidomethyl polystyrene resin (PS-HOBt, 0.98 mmol g^{-1}) was purchased from Argonaut technologies. PGHS-1, PGHS-2 and PPHP were from Cayman Chemicals, Michigan, USA. All other reagents and solvents, purchased from Sigma-Aldrich and Lancaster, were of the highest possible quality and were used without further purification. The elemental analyses were performed by SGS MULTILAB (Evry, France).

Synthesis

Three arrays of 10 anthranilic hydroxamic acids were prepared on a QUEST 210 ASW Synthesiser (Argonaut Technologies, Cardiff, UK). The reactions were performed under an atmosphere of nitrogen. Percentage yields were estimated from the isolated weight of each hydroxamic acid and are based on the amount of hydroxylamine used (0.9 equivalents of the PS-HOBt resin initial loading). Chromatographic analysis and purification were performed on a PerSeptive Biosystems BioCAD SPRINT Perfusion Chromatography Workstation using POROS 20R2 reverse-phase chromatography packing (column: 4.6 mmD × 100 mmL, 1.7 ml, self-packed; A mobile phase: 0.1% TFA in water; B mobile phase: 0.1% TFA in acetonitrile; gradient: 2 to 60% B in 18 column volumes at 7 ml min^{-1} flow rate, for analysis) (column: 10 mmD × 100 mmL, 7.9 ml, self-packed; A mobile phase: 0.1% TFA in water; B mobile phase: 0.1% TFA in acetonitrile; gradient: 2 to 60% B in 20 column volumes at 12 ml min^{-1} flow rate, for purification). Purities were ascertained from the percent area of the hydroxamic acid relative to the total area of all UV absorbing components (235 nm and 280 nm). The carboxylic acid precursors, the main impurities in the current work, are predicted to have absorbance maxima analogous to the corresponding hydroxamic acids. Liquid chromatography-mass spectrometry experiments were performed on a Quattro Micro quadrupole electrospray mass spectrometer (Micromass, Waters Corp., USA): 10 μL of the samples were injected in 300 μL of acetonitrile : water (60 : 40, v/v). The mass spectrometry data were acquired both in positive and negative ion modes. Nuclear magnetic resonance spectra were recorded on a Bruker DPX400 NMR.

The synthesis of the anthranilic hydroxamic acids was carried out as previously reported[22] on a 0.245 mmol scale for the resin, except that the loading and the coupling step were performed for 2 h and 4 h respectively. 0.9 Equivalent of free hydroxylamine[41] relative to the initial loading of the PS-HOBt resin was used. The hydroxamic acids were obtained in quantitative yields and with high purities. Compounds **4–10**, **13–21**, **24–26** and **28–30** are reported for the first time.

N,*N*′-Diisopropylurea was synthesised as follow: a solution of 500 mg (4 mmol) of *N*,*N*′-diisopropylcarbodiimide in 2.5 ml of THF was added to 5 ml of a 1 M NaOH solution and the resulting mixture was vigorously stirred for 2 h at room temperature. The organic solvent was evaporated under reduced pressure and the aqueous solution was stored at 4 °C overnight. The crystals formed were isolated by filtration, rinsed with water and dried under vacuum to give 57 mg (12%) of product. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 5.5–5.48 (2H, d, NH), 3.64 (2H, hep, J = 6.52 Hz, CH), 1.01–0.99 (12H, d, J = 6.52 Hz, CH_3). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 156.76, 40.66, 23.30.

TMPD assay for PGHS peroxidase activity

Peroxidase activity was measured by monitoring the PGHS enzyme catalysed oxidation of the reducing co-substrate *N*,*N*,*N*′,*N*′-tetra-*p*-methylphenylenediamine dihydrochloride (TMPD), at 611 nm.[25] Reactions were carried out at 37 °C in a 200 μL cuvette. Compounds were dissolved in DMSO at various concentrations (0.01 μM to 1 mM) and were incubated with 23 nM of PGHS-1 or 365 nM PGHS-2 for one minute at 37 °C in a 200 μL solution of 0.1 M Tris pH 8.0 and 1 μM haematin (final DMSO concentration <1.0%). The reaction was initiated with the addition of 200 μM TMPD followed by 200 μM H_2O_2. The rate of TMPD oxidation recorded in the first 4 seconds of the reaction was taken as a measure of the enzyme's initial velocity. Enzyme activity was normalised to the activity of solutions containing the vehicle alone.

Each experiment was carried out in duplicate ($n \geq 3$). Initial velocities were converted to percentage of maximal activity and plotted against the $\log_{10}$ of inhibitor concentrations (μM). The titration curve was fitted to a four-parameter non-linear regression equation (SigmaPlot 8.0, SPSS Inc.) of the form: $y = \min + (\max - \min)/[1 + 10\exp(\log IC_{50} - x)]$ where max and min are asymptotes, x is the logarithm of the inhibitor concentration and $\log IC_{50}$ is the inflection point. Only data with an R^2 value greater than 0.900 were considered ($n = 3$). The corresponding carboxylic acids were tested and showed no activity (*i.e.* $IC_{50} > 1000$ μM).

PPHP assay for PGHS peroxidase activity

The PPHP assay is based on the chromatographic analysis of the of conversion of a lipid peroxide into its corresponding alcohol and has been adapted from a reported procedure.[27] Purified ovine PGHS-1 (4.3 nM) was pre-incubated for 3 minutes with 200 μM of each compound in 500 μL of 100 mM Tris buffer (pH 8.0), 1 μM haematin at 37 °C (final DMSO concentration <1.0%). Reactions were initiated by adding 100 μM PPHP (stock in ethanol) and incubated at 37 °C for 3 minutes (final ethanol concentration <0.5%). Reactions were terminated by C_{18} reverse-phase extraction. The C_{18} columns (Empore C18-HD, 3 M) were pre-equilibrated with 1 ml H_2O, reaction mixtures were then added and the columns were washed with 4 ml H_2O, and eluted in 1 ml methanol. PPHP and PPA were separated using reverse-phase HPLC on a C_{12} column (Synergi 4 μm MAX-RP; 150 mmL × 4.6 mmD; Phenomenex). Each sample (200 μl) was injected using a BioCAD SPRINT HPLC workstation (Applied Biosystems) and isocratically eluted with 65% methanol–H_2O at a flow rate of 1.0 ml min^{-1} with detection at 254 nm. Under these conditions all anthranilic hydroxamic acids eluted in less then 7.0 minutes, while PPA and PPHP eluted at 7.6 and 8.7 minutes respectively, with equal peak absorbance integrals. Product formation was expressed as the ratio of the integrated absorbance of PPA to total substrate and product [*i.e.* PPA/(PPA + PPHP)]. Background conversion due to heme (average = 0.38) was subtracted from each reading. Maximal activity, found using an excess of enzyme (34 nM), was used to convert each ratio to percentage of maximal activity. Phenol showed 42% ± 12% of maximal conversion while the control (no phenol) showed 24% ± 12% conversion. Assays were performed in duplicate with $n = 2$ to 4.

Characterization of AHA derivatives

Anthranilic hydroxamic acid (3). Isolated as a light brown solid in 94% yield, 31 mg. Found: C, 55.32; H, 5.28; N, 18.27. Calc. for $C_7H_8N_2O_2$: C, 55.26; H, 5.30; N, 18.41%. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.89 (1H, br s, OH), 8.85 (1H, br s, NH), 7.31–7.29 (1H, d, J = 8 Hz, ArH6), 7.13–7.12 (1H, m,

$J = 7$ Hz, 8 Hz, ArH5), 6.69–6.67 (1H, d, $J = 8$ Hz, ArH3), 6.49–6.47 (1H, m, $J = 7$ Hz, 8 Hz, ArH4), 6.23 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 166.89, 149.23, 131.97, 127.93, 116.57, 115.08, 113.05. Purity (RP-HPLC): 97% ($t_r = 1.05$); m/z (ES) calc. for $C_7H_8N_2O_2$ 151.2, found 151.2.

3-Chloroanthranilic hydroxamic acid (4). Isolated as a cream solid in 92% yield, 38 mg. ^1H NMR (DMSO-d_6, 400 MHz): δ 11.14 (1H, s, OH), 9.05 (1H, s, NH), 7.38–7.36 (1H, dd, $J =$ 1 Hz, $J = 7.52$ Hz, ArH6), 7.34–7.32 (1H, d, $J = 7.52$ Hz, ArH4), 6.58–6.56 (1H, m, $J = 7.52$ Hz, 8 Hz, ArH5), 6.35 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 165.93, 157.21, 144.75, 131.60, 126.57, 118.87, 115.43. Purity (RP-HPLC): 95% ($t_r = 2.52$); m/z (ES) calc. for $C_7H_7N_2ClO_2$ 185.6, found 185.1.

3-Methoxyanthranilic hydroxamic acid (5). Isolated as a brown oil in 81% yield, 30 mg. ^1H NMR (DMSO-d_6, 400 MHz): δ 10.93 (1H, br s, OH), 9.21 (1H, br s, NH), 7.00–6.98 (1H, dd, $J = 1$ Hz, $J = 7$ Hz, ArH6), 6.89–6.87 (1H, d, $J = 8$ Hz, ArH4), 6.50–6.48 (1H, m, $J = 7$ Hz, 8 Hz, ArH5), 3.79–3.77 (3H, s, $J = 5$ Hz, $ArOCH_3$), 5.94 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 166.75, 146.83, 139.16, 119.20, 114.24, 112.65, 111.92, 55.54. Purity (RP-HPLC): 90% ($t_r = 1.12$); m/z (ES) calc. for $C_8H_{10}N_2O_3$ 181.2, found 181.1.

3-Trifluoromethylanthranilic hydroxamic acid (6). Isolated as a cream solid in 85% yield, 41 mg. ^1H NMR (DMSO-d_6, 400 MHz): δ 11.23 (1H, br s, OH), 9.14 (1H, br s, NH), 7.58–7.57 (1H, d, $J = 7.52$ Hz, ArH6), 7.51–7.49 (1H, d, $J = 7.48$ Hz, ArH4), 6.68–6.66 (1H, m, $J = 7.48$, 7.52 Hz, ArH5), 6.51 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 169.28, 165.82, 145.93, 132.62, 129.39, 124.90, 115.88, 114.68. Purity (RP-HPLC): 96% ($t_r = 2.88$); m/z (ES) calc. for $C_8H_7N_2F_3O_2$ 219.2, found 219.2.

4-Chloroanthranilic hydroxamic acid (7). Isolated as a cream solid in 73% yield, 30 mg. ^1H NMR (DMSO-d_6, 400 MHz): δ 10.98 (1H, br s, OH), 9.39 (1H, br s, NH), 7.33–7.31 (1H, m, $J = 3.44$ Hz, $J = 8.20$ Hz, ArH6), 6.77–6.76 (1H, m, $J =$ 2.04 Hz, ArH3), 6.50–6.48 (1H, m, $J = 6.8$ Hz, $J = 2.04$ Hz ArH5), 6.52 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 166.00, 150.60, 136.04, 129.31, 115.35, 114.80, 111.72. Purity (RP-HPLC): 94% ($t_r = 1.97$); m/z (ES) calc. for $C_7H_7N_2ClO_2$ 185.6, found 185.2.

4-Fluoroanthranilic hydroxamic acid (8). Isolated as a cream solid in 85% yield, 32 mg. ^1H NMR (DMSO-d_6, 400 MHz): δ 10.94 (1H, s, OH), 9.58 (1H, s, NH), 7.39–7.37 (1H, m, $J =$ 6.84 Hz, $J = 8.88$ Hz, ArH6), 6.49–6.46 (1H, m, $J = 12.28$ Hz, $J = 2.72$Hz, ArH3), 6.30–6.28 (1H, m, $J = 8.88$ Hz, $J = 2.72$ H ArH5), 6.58 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 165.55, 163.11, 151.82, 130.04, 124.91, 109.64, 101.57. Purity (RP-HPLC): 90% ($t_r = 1.27$); m/z (ES) calc. for $C_7H_7N_2FO_2$ 169.1, found 169.2.

4-Nitroanthranilic hydroxamic acid (9). Isolated as a light brown solid in 62% yield, 27 mg. ^1H NMR (DMSO-d_6, 400 MHz): δ 11.28 (1H, br s, OH), 9.17 (1H, br s, NH), 7.58–7.58 (1H, m, $J = 2$ Hz, ArH6), 7.53–7.51 (1H, m, $J = 8.52$ Hz, ArH3), 7.28–7.25 (1H, m, $J = 8.52$ Hz, $J = 2.52$H, ArH5), 6.72 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 166.74, 156.90, 149.33, 133.03, 129.12, 109.81, 108.46. Purity (RP-HPLC): 90% ($t_r = 1.60$); m/z (ES) calc. for $C_7H_7N_2KO_4$ (*i.e.* K^+ salt) 235.2, found 235.3.

5-Acetamidoanthranilic hydroxamic acid (10). Isolated as a pale cream solid in 82% yield, 37 mg. ^1H NMR (DMSO-d_6, 400 MHz): δ 9.67 (1H, s, NHOH), 8.71 (1H, s, NH), 7.48–7.47 (1H, S, $J = 2$ Hz, ArH6), 7.30–7.28 (1H, d, $J = 8.8$ Hz, $J =$ 2.76 Hz, ArH4), 6.64–6.62 (1H, d, $J = 8.8$ Hz, ArH3), 5.86 (2H, br s, NH_2), 1.96 (3H, s, $COCH_3$). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 170.85, 167.76, 147.61, 127.73, 124.39, 119.59, 116.23, 107.01, 23.31. Purity (RP-HPLC): 98% ($t_r = 1.05$); m/z (ES) calc. for $C_9H_{11}N_3O_3$ 208.2, found 208.2.

5-Bromoanthranilic hydroxamic acid (11). Isolated as a light brown solid in 57% yield, 29 mg. ^1H NMR (DMSO-d_6, 400 MHz): δ 11.05 (1H, s, OH), 8.96 (1H, s, NH), 7.46–7.45 (1H, d, $J = 2.52$ Hz, ArH6), 7.27–7.25 (1H, m, $J = 8.52$ Hz, $J =$ 2.52 Hz, ArH4), 6.69–6.67 (1H, d, $J = 9$ Hz, ArH3), 6.40 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 165.54, 148.48, 134.05, 129.63, 118.26, 114.56, 104.97. Purity (RP-HPLC): 88% ($t_r = 1.93$); m/z (ES) calc. for $C_7H_7N_2BrO_2$ 230.1, found 230.9.

5-Chloroanthranilic hydroxamic acid (12). Isolated as a cream solid in 70% yield, 29 mg. ^1H NMR (DMSO-d_6, 400 MHz): δ 11.06 (1H, s, OH), 8.98 (1H, s, NH), 7.35–7.34 (1H, d, $J = 2.52$ Hz, ArH6), 7.16–7.13 (1H, m, $J = 9$ Hz, $J = 2.48$ Hz, ArH4), 6.73–6.71 (1H, m, $J = 8.52$ Hz, ArH3), 6.38 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 162.31, 148.16, 138.94, 131.30, 126.82, 117.82, 113.86. Purity (RP-HPLC): 95% ($t_r = 1.45$); m/z (ES) calc. for $C_7H_7N_2ClO_2$ 185.6, found 185.2.

5-Fluoroanthranilic hydroxamic acid (13). Isolated as a cream solid in 53% yield, 32 mg. ^1H NMR (DMSO-d_6, 400 MHz): δ 10.86 (1H, br s, OH), 9.20 (1H, br s, NH), 7.17–7.14 (1H, m, $J = 10$ Hz, $J = 3$ Hz, ArH6), 7.06–7.04 (1H, m, $J =$ 8.56 Hz, $J = 3$ Hz, ArH4), 6.73–6.71 (1H, m, $J = 9$ Hz, $J = 5$ Hz, ArH3), 6.15 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 153.72, 145.99, 139.76, 118.84, 117.36, 113.19, 106.92. Purity (RP-HPLC): 95% ($t_r = 1.05$); m/z (ES) calc. for $C_7H_7N_2FO_2$ 169.1, found 169.1.

5-Hydroxyanthranilic hydroxamic acid (14). Isolated as a brown solid in 76% yield, 27 mg. ^1H NMR (DMSO-d_6, 400 MHz): δ 10.19 (1H, s, OH), 8.85 (1H, s, NH), 8.71 (1H, s, OH, ArH5), 6.74–6.73 (1H, s, $J = 2.52$ Hz, ArH6), 6.70–6.68 (1H, m, $J = 8.52$ Hz, $J = 2.52$ Hz, ArH4), 6.59–6.57 (1H, d, $J = 8.56$ Hz, ArH3), 6.59 (2H, s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 162.32, 147.35, 139.21, 119.67, 117.65, 113.45, 106.93. Purity (RP-HPLC): 90% ($t_r = 1.05$); m/z (ES) calc. for $C_7H_8N_2O_3$ 167.2, found 167.1.

5-Iodoanthranilic hydroxamic acid (15). Isolated as a black solid in 85% yield, 49 mg. ^1H NMR (DMSO-d_6, 400 MHz): δ 11.02 (1H, br s, OH), 8.94 (1H, br s, NH), 7.58 (1H, s, ArH6), 7.39–7.37 (1H, d, $J = 8.52$ Hz, ArH4), 6.58–6.56 (1H, d, $J =$ 8.56 Hz, ArH3), 6.39 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 165.50, 150.91, 139.53, 135.38, 118.71, 115.50, 106.93. Purity (RP-HPLC): 93% ($t_r = 2.83$); m/z (ES) calc. for $C_7H_7N_2IO_2$ 277.0, found 277.0.

5-Methoxyanthranilic hydroxamic acid (16). Isolated as a brown solid in 63%, 24 mg. ^1H NMR (DMSO-d_6, 400 MHz): δ 10.71 (1H, br s, OH), 8.89 (1H, br s, NH), 6.91–6.90 (1H, m, $J =$ 3 Hz, ArH6), 6.84–6.82 (1H, m, $J = 8.52$ Hz, $J = 3$ Hz, ArH4), 6.67–6.65 (1H, m, $J = 9$ Hz, ArH3), 3.66 (3H, $ArOCH_3$). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 149.29, 143.34, 139.05, 119.31, 117.60, 111.39, 106.94, 55.41. Purity (RP-HPLC): 94% ($t_r =$ 1.05); m/z (ES) calc. for $C_8H_{10}N_2O_3$ 181.2, found 181.1.

5-Methylanthranilic hydroxamic acid (17). Isolated as a cream solid in quantitative yield, 36 mg. ^1H NMR (DMSO-d_6, 400 MHz): δ 10.85 (1H, br s, OH), 8.84 (1H, br s, NH), 7.14 (1H, s, ArH6), 6.97–6.95 (1H, m, $J = 8.52$ Hz, $J =$ 2 Hz, ArH4), 6.63–6.60 (1H, m, $J = 8.52$ Hz, ArH3), 2.13 (3H, s, $ArCH_3$), 6.01 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz,): δ 162.33, 146.88, 132.36, 127.57, 123.04, 116.34, 113.19, 20.00. Purity (RP-HPLC): 96% ($t_r = 1.07$); m/z (ES) calc. for $C_8H_{10}N_2O_2$ 165.2, found 165.2. Purified by RP-HPLC (Experimental section) Found: C, 41.92; H, 3.88; N, 9.35. Calc. for $C_8H_{10}N_2O_2 \cdot TFA \cdot 0.5\ H_2O$: C, 41.52; H, 4.18; N, 9.68%.

5-Nitroanthranilic hydroxamic acid (18). Isolated as a yellow solid in 98% yield, 40 mg. ^1H NMR (DMSO-d_6, 400 MHz):

δ 11.38 (1H, br s, OH), 9.15 (1H, br s, NH), 8.32–8.31 (1H, m, J = 2.48 Hz, ArH6), 8.03–8.01 (1H, m, J = 9.04 Hz, J = 2.52 Hz, ArH4), 6.82–6.80 (1H, d, J = 9.04 Hz, ArH3), 7.61 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz,): δ 154.89, 139.35, 134.84, 127.36, 125.18, 115.60, 111.30. Purity (RP-HPLC): 81% (t_r = 1.62); m/z (ES) calc. for $C_7H_7N_3O_4$ 196.2, found 196.1.

6-Chloroanthranilic hydroxamic acid (19). Isolated as a light grey solid in 74% yield, 29 mg. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.98 (1H, br s, OH), 9.27 (1H, br s, NH), 7.06–7.04 (1H, m, J = 8.2, J = 7.52 Hz, ArH4), 6.64–6.62 (1H, d, J = 8.2 Hz, ArH5), 6.59–6.57 (1H, d, J = 7.52Hz, ArH3), 5.20 (2H, s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz,): δ 162.00, 147.84, 130.96, 130.49, 118.89, 116.03, 113.55. Purity (RP-HPLC): 90% (t_r = 1.10); m/z (ES) calc. for $C_7H_7N_2ClO_2$ 185.6, found 185.2.

6-Fluoroanthranilic hydroxamic acid (20). Isolated as a cream solid in 69% yield, 39 mg. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.90 (1H, s, OH), 9.17 (1H, br s, NH), 7.10–7.08 (1H, q, J = 8.04, 9.52 Hz, ArH4), 6.52–6.50 (1H, d, J = 8.04 Hz, ArH5), 6.34–6.31 (1H, t, J = 9.52Hz, ArH3), 5.71 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 161.17, 158.90, 149.56, 131.15, 111.15, 105.78, 101.70. Purity (RP-HPLC): 90% (t_r = 1.18); m/z (ES) calc. for $C_7H_7N_2FO_2$ 169.1, found 169.2.

6-Methylanthranilic hydroxamic acid (21). Isolated as a cream solid in 74% yield, 25 mg. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.79 (1H, br s, OH), 9.23 (1H, br s, NH), 6.95–6.94 (1H, m, J = 8 Hz, 7.52 Hz, J = 2.52 Hz, ArH4), 6.51–6.49 (1H, d, J = 8 Hz, ArH5), 6.39–6.37 (1H, d, J = 7.52 Hz, ArH3), 2.14–2.14 (3H, d, J = 2.48 Hz, $ArCH_3$), 4.85 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz,): δ 164.84, 146.06, 135.23, 129.24, 120.19, 117.68, 112.57, 19.48. Purity (RP-HPLC): 87% (t_r = 1.05); m/z (ES) calc. for $C_8H_{10}N_2O_2$ 165.2, found 165.1.

3,5-Dichloroanthranilic hydroxamic acid (22). Isolated as grey crystals in 98% yield, 48 mg. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 11.27 (1H, s, OH), 9.16 (1H, s, NH), 7.51–7.51 (1H, m, J = 2 Hz, ArH6), 7.51–7.40 (1H, m, J = 2 Hz, ArH4), 6.47 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 164.72, 143.95, 130.84, 126.25, 119.98, 117.99, 115.63. Purity (RP-HPLC): 93% (t_r = 3.38); m/z (ES) calc. for $C_7H_6N_2Cl_2O_2$ 220.0, found 219.1. Purified by RP-HPLC (Experimental section) Found: C, 38.11; H, 2,71; N, 12.53; Cl, 31.08. Calc. for $C_7H_6Cl_2N_2O_2$: C, 38.04; H, 2.74; N, 12.67; Cl, 32.08%.

3,5-Dibromoanthranilic hydroxamic acid (23). Isolated as a cream solid in 85% yield, 58 mg. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 11.20 (1H, br s, OH), 9.35 (1H, br s, NH), 7.72–7.71 (1H, d, J = 2.04 Hz, ArH6), 7.72–7.53 (1H, m, J = 2.04 Hz ArH4), 6.42 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz,): δ 164.36, 145.04, 136.27, 129.54, 116.26, 110.08, 105.28. Purity (RP-HPLC): 88% (t_r = 3.72); m/z (ES) calc. for $C_7H_6N_2Br_2O_2$ 309.0, found 309.0.

3,5-Diiodoanthranilic hydroxamic acid (24). Isolated as a white solid in 79% yield, 71 mg. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 11.19(1H, br s, OH), 9.11 (1H, br s, NH), 7.96 (1H, s, ArH6), 7.60 (1H, s, ArH4), 6.31 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 164.40, 149.34, 147.60, 140.11, 135.77, 124.91, 116.32. IR (KBr) cm^{-1}; 3438, 3343, 3195, 1640. Purity (RP-HPLC): 93% (t_r = 4.17); m/z (ES) calc. for $C_7H_6N_2I_2O_2$ 403.9, found 403.0. Purified by RP-HPLC (Experimental section) Found: C, 21.38; H, 1.58; N, 6.54; I, 62.31. Calc. for $C_7H_6I_2N_2O_2$: C, 20.81; H, 1.50; N, 6.93; I, 62.83%.

3,5-Dimethylanthranilic hydroxamic acid (25). Isolated as a cream solid in 99% yield, 37 mg. ^{1}H NMR (DMSO-d_6, 400 MHz,): δ 10.91 (1H, br s, OH), 8.86 (1H, br s, NH), 7.04 (1H, s, ArH6), 6.91 (1H, s, ArH4), 2.12 (3H, s, ArC5, CH_3), 2.05 (3H, s, ArC3, CH_3,), 5.91(2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 167.28, 144.61, 138.94, 133.39, 125.42, 123.25, 113.31, 20.02, 17.55. Purity (RP-HPLC): 96% (t_r = 1.23); m/z (ES) calc. for $C_9H_{12}N_2O_2$ 179.2, found 179.1.

3-Bromo-5-methylanthranilic hydroxamic acid (26). Isolated as a cream solid in 78% yield, 40 mg. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 11.10 (1H, br s, OH), 9.04 (1H, s, NH), 7.37 (1H, br s, ArH6), 7.20 (1H, s, ArH4), 2.15 (3H, s, ArC5, CH_3), 6.04 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 165.89, 143.25, 135.16, 127.66, 125.09, 115.43, 109.48, 19.50. Purity (RP-HPLC): 94% (t_r = 3.22); m/z (ES) calc. for $C_8H_9N_2BrO_2$ 244.1, found 243.1.

3,4,5-Trimethoxyanthranilic hydroxamic acid (27). Isolated as a dark brown solid in 97% yield, 49 mg. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.95 (1H, br s, OH), 8.88 (1H, br s, NH), 6.84 (1H, s, ArH6), 3.78 (3H, s, ArC5, CH_3), 3.71 (12H, br s, ArC3, ArC4, ArC5, OCH_3), 5.83 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz,): δ 166.45, 145.14, 142.59, 140.31, 138.97, 107.37, 106.93, 60.37, 59.98, 56.33. Purity (RP-HPLC): 87% (t_r = 1.43); m/z (ES) calc. for $C_{10}H_{17}N_2O_2$ 241.2, found 241.0.

3,4,5,6-Tetrafluoroanthranilic hydroxamic acid (28). Isolated as a brown solid in 60% yield, 30 mg. ^{1}H NMR (DMSO-d6, 400 MHz): δ 11.20 (1H, br s, OH), 9.41 (1H, br s, NH), 5.84 (2H, s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 158.35, 139.29, 138.99, 133.47, 124.96, 106.99, 103.15. Purity (RP-HPLC): 77% (t_r = 2.02); m/z (ES) calc. for $C_7H_4N_2F_4O_2$ 223.1, found 223.1.

4,5-Difluoroanthranilic hydroxamic acid (29). Isolated as a cream solid in 87% yield, 46 mg. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 13.39 (1H, br s, OH), 11.93 (1H, br s, NH), 7.77 (1H, s, ArH6), 7.76 (1H, s, ArH2), 9.70 (2H, s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 165.94, 157.11, 132.71, 124.75, 122.37, 115.12, 105.53. Purity (RP-HPLC): 98% (t_r = 1.35); m/z (ES) calc. for $C_7H_6N_2F_2O_2$ 187.1, found 187.2.

4,5-Dimethoxyanthranilic hydroxamic acid (30). Isolated as a brown solid in 83% yield, 39 mg. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 10.74 (1H, br s, OH), 8.69 (1H, s, NH), 6.94 (1H, s, ArH6), 6.31 (1H, s, ArH3), 3.70 (3H, s, ArC5, OCH_3), 3.64 (3H, s, ArH4, OCH_3), 6.20 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 167.08, 152.59, 145.86, 138.95, 111.29, 102.90, 99.86, 56.26, 55.07. Purity (RP-HPLC): 92% (t_r = 1.07); m/z (ES) calc. for $C_9H_{12}N_2O_4$ 211.2, found 211.2.

Nicotinic anthranilic hydroxamic acid (31). Isolated as a white solid in 88% yield, 28 mg. ^{1}H NMR (DMSO-d_6, 400 MHz): δ 11.20 (1H, br s, OH), 9.07 (1H, br s, NH), 8.07–8.06 (1H, m, J = 5 Hz, J = 1.52 Hz, ArH5), 7.77–7.75 (1H, d, J = 7.52 Hz, ArH6), 6.60–6.58 (1H, m, J = 7.56 Hz, J = 4.52 Hz, ArH3), 7.04 (2H, br s, NH_2). ^{13}C NMR (DMSO-d_6, 100 MHz): δ 149.96, 140.34, 136.43, 111.35, 108.55, 106.94. Purity (RP-HPLC): 98% (t_r = 1.07); m/z (ES) calc. for $C_6H_7N_3O_2$ 152.1, found 152.1.

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References

1 R. J. Kulmacz, W. A. van der Donk and A. L. Tsai, *Prog. Lipid Res.*, 2003, **42**, 377–404.
2 R. M. Garavito, M. G. Malkowski and D. L. DeWitt, *Prostaglandins Other Lipid Mediat.*, 2002, **68–69**, 129–152.
3 R. M. Garavito and A. M. Mulichak, *Annu. Rev. Biophys. Biomol. Struct.*, 2003, **32**, 183–206.

4 R. J. A. Helliwell, L. F. Adams and M. D. Mitchell, *Prostaglandins Leukot. Essent. Fatty Acids*, 2004, **70**, 101–113.
5 W. L. Smith, D. L. DeWitt and R. M. Garavito, *Annu. Rev. Biochem.*, 2000, **69**, 145–182.
6 N. V. Chandrasekharan, H. Dai, K. L. Roos, N. K. Evanson, J. Tomsik, T. S. Elton and D. L. Simmons, *Proc. Natl. Acad. Sci. USA*, 2002, **99**, 13926–31.
7 D. Picot, P. J. Loll and R. M. Garavito, *Nature*, 1994, **367**, 243–249.
8 D. L. DeWitt, E. A. El-Harith, S. A. Kraemer, M. J. Andrews, E. F. Yao, R. L. Armstrong and W. L. Smith, *J. Biol. Chem.*, 1990, **265**, 5192–8.
9 K. Mizuno, S. Yamamoto and W. E. M. Lands, *Prostaglandins*, 1982, **23**, 743–757.
10 P. J. Marshall and R. J. Kulmacz, *Arch. Biochem. Biophys.*, 1988, **266**, 162–170.
11 R. C. Forde and D. J. Fitzgerald, *Circulation*, 1997, **95**, 787–789.
12 V. B. O'Donnell, B. Coles, M. J. Lewis, B. C. Crews, L. J. Marnett and B. A. Freeman, *J. Biol. Chem.*, 2000, **275**, 38239–38244.
13 R. J. Kulmacz, R. B. Pendleton and W. E. Lands, *J. Biol. Chem.*, 1994, **269**, 5527–36.
14 M. Ouellet, S. M. Aitken, A. M. English and M. D. Percival, *Arch. Biochem. Biophys.*, 2004, **431**, 107–118.
15 L. Szewczuk, L. Forti, L. A. Stivala and T. M. Penning, *J. Biol. Chem.*, 2004, **279**, 22727–22737.
16 P. J. Loll, C. T. Sharkey, S. J. O'Connor, C. M. Dooley, E. O'Brien, M. Devocelle, K. B. Nolan, B. S. Selinsky and D. J. Fitzgerald, *Mol. Pharmacol.*, 2001, **60**, 1407–1413.
17 C. M. Dooley, M. Devocelle, B. McLoughlin, K. B. Nolan, D. J. Fitzgerald and C. T. Sharkey, *Mol. Pharmacol.*, 2003, **63**, 450–455.
18 M. Ikeda-Saito, D. A. Shelley, L. Lu, K. S. Booth, W. S. Caughey and S. Kimura, *J. Biol. Chem.*, 1991, **266**, 3611–3616.
19 H. Itakura, Y. Oda and K. Fukuyama, *FEBS Lett.*, 1997, **412**, 107–110.
20 A. Henriksen, D. J. Schuller, K. Meno, K. G. Welinder, A. T. Smith and M. Gajhede, *Biochemistry*, 1998, **37**, 8054–8060.
21 C. Indiani, E. Santoni, M. Becucci, A. Boffi, K. Fukuyama and G. Smulevich, *Biochemistry*, 2003, **42**, 14066–14074.
22 M. Devocelle, B. M. McLoughlin, C. T. Sharkey, D. J. Fitzgerald and K.B. Nolan, *Org. Biomol. Chem.*, 2003, **1**, 850–853.
23 L. Gouilleux, J. Fehrentz, A. F. Winternitz and J. Martinez, *Tetrahedron Lett.*, 1996, **37**, 7031–7034.
24 A. Golebiowski and S. Klopfenstein, *Tetrahedron Lett.*, 1998, **39**, 3397–3400.
25 S. C. C. Tam, D. H. S. Lee, E. Y. Wang, D. G. Munroe and C. Y. Lau, *J. Biol. Chem.*, 1995, **270**, 13948–13955.
26 C. M. Dooley, PhD Thesis, National University of Ireland, 2003.
27 C. M. Markey, A. Alward, P. E. Weller and L. J. Marnett, *J. Biol. Chem.*, 1987, **262**, 6266–6279.
28 Consistent with this is the fact that *N*-methyl hydroxamate 3,5-diiodo AHA (the *N*-methyl derivative of the most active inhibitor) was an ineffective inhibitor.
29 R. I. Walter, *J. Am. Chem. Soc.*, 1966, **88**, 1930.
30 R. I. Walter, *J. Am. Chem. Soc.*, 1966, **88**, 1923.
31 F. G. Bordwell and W.-Z. Liu, *J. Am. Chem. Soc.*, 1996, **118**, 10819.
32 Z. Li and J.-P. Cheng, *J. Org. Chem.*, 2003, **68**, 7350–7360.
33 P. Kazmierczak and L. Skulski, *Synthesis*, 1998, **12**, 1721–1723.
34 J. J. P. Stewart, *J. Comput. Chem.*, 1989, **10**, 209–220.
35 P. Jones, *J. Biol. Chem.*, 2001, **276**, 13791–13796.
36 G. M. Morris, D. S. Goodsell, R. S. Halliday, R. Huey, W. E. Hart, R. K. Belew and A. J. Olson, *J. Comput. Chem.*, 1998, **19**, 1639–1662.
37 K. Gupta, B. S. Selinsky, C. J. Kaub, A. K. Katz and P. J. Loll, *J. Mol. Biol.*, 2004, **335**, 503–518.
38 A. J. Chubb, E. Moman, D. J. Fitzgerald and K. B. Nolan, The Productive Conformation of PGG_2 at the Peroxidase Site of Prostaglandin Endoperoxidase H_2 Synthase, submitted.
39 Molecular Operating Environment, version 2004.03, Chemical Computing Group Inc., Montreal.
40 S. Shahapurkar, T. Pandya, N. Kawathekar and S. C. Chaturvedi, *Eur. J. Med. Chem.*, 2004, **39**, 383–388.
41 E. Thouin and W. D. Lubell, *Tetrahedron Lett.*, 2000, **41**, 457–460.