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Inactivation of bovine plasma amine oxidase by haloallylamines

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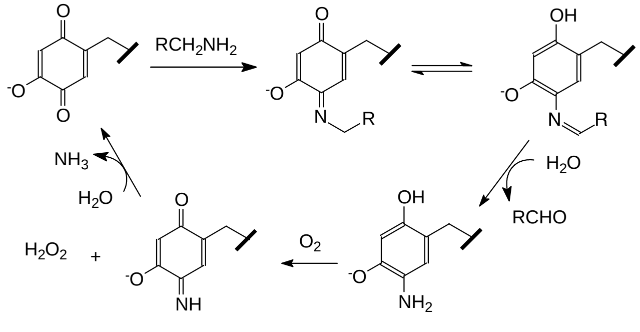
Abstract—Various 2- and 3-haloallylamines were synthesized and evaluated as inhibitors of the quinone-dependent bovine plasma amine oxidase (BPAO). 3-Haloallylamines, which were previously found to be good inhibitors of the flavin-dependent mitochondrial monoamine oxidase (MAO), exhibited a time-dependent inactivation of BPAO, with the 2-phenyl analogs being more potent than the 2-methyl analogs. No plateau of enzyme activity loss was observed, suggestive of a lack of competitive partitioning to normal turnover. The (*E*)- and (*Z*)-2-phenyl-3-fluoro analogs were the most potent (low μM IC_{50}s), with the corresponding 3-bromo and 3-chloro analogs being >10-fold less potent. In each case, the *Z*-isomers were more potent than the *E*-isomers, the reverse of the configurational inhibitory preference observed with MAO. In contrast to the 2-phenyl analogs, 3-phenyl-2(or 3)-chloroallylamines displayed a partitioning behavior, consistent with these being both substrates and inactivators of BPAO.

1. Introduction

The copper-containing amine oxidases (CAOs, EC 1.4.3.6) are a ubiquitous family of enzymes, occurring in bacteria, yeast, plants, and mammals, that catalyze the oxidative deamination of primary amines to give the corresponding aldehydes.[1–3] In prokaryotic organisms, these enzymes play major roles in nutrient catabolism, whereas in plants and mammals, they play important roles in metabolizing biogenic amines (including mono-, di-, and polyamines and neurotransmitters) involved in growth, cell division, differentiation, and the stress response, as well as in metabolism of xenobiotic amines, in some cases with cytotoxic consequences.

Unlike the flavin-dependent mitochondrial monoamine oxidase (MAO), which can oxidize primary, secondary, and tertiary amines, the CAOs have a nearly strict preference for oxidation of primary amines. Most enzymes in this family contain the trihydroxyphenylalanine quinone (TPQ) cofactor,[4] derived post-translationally from an active site tyrosine residue.[5] This quinone cofactor, along with active site general acid–base catalysis, achieves a pyridoxal-like transamination of the primary amine to give the aldehyde. The mechanism involves formation of quinone cofactor-substrate Schiff base, general base-mediated isomerization to a product Schiff base, and hydrolysis of the latter to generate product aldehyde and the reductively aminated form of the cofactor (an aminoresorcinol). The quinone cofactor is recovered by O_2-dependent oxidation of reduced cofactor, releasing ammonia and H_2O_2 (Scheme 1).[6] Driven largely by the recent discovery that one of the three TPQ-dependent human gene products known as semicarbazide-sensitive amine oxidase (SSAO) is identical with the human vascular adhesion protein-1 (HVAP-1),[7,8] the CAOs have become potential therapeutic targets for intervening in a number of disease states, including inflammatory diseases, fibrotic diseases (arthritis and atherosclerosis), diabetes, cardiovascular disease, and immune dysfunction diseases such as multiple sclerosis.



Scheme 1.

Keywords: Copper amine oxidase; Enzyme inhibition; Haloallylamine.

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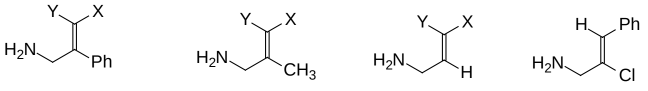


1a: X = Cl, Y = H;
1b: X = H, Y = Cl;
1c: X = Br, Y = H;
1d: X = H, Y = Br;
1e: X = F, Y = H;
1f: X = H, Y = F;
1g: X = F, Y = F.

1h: X = Br, Y = H:
1i: X = H, Y =Br.

1j: X = Cl, Y = Ph;
1k: X = Ph, Y = Cl.

1l

Figure 1. Structures of compounds evaluated in this study.

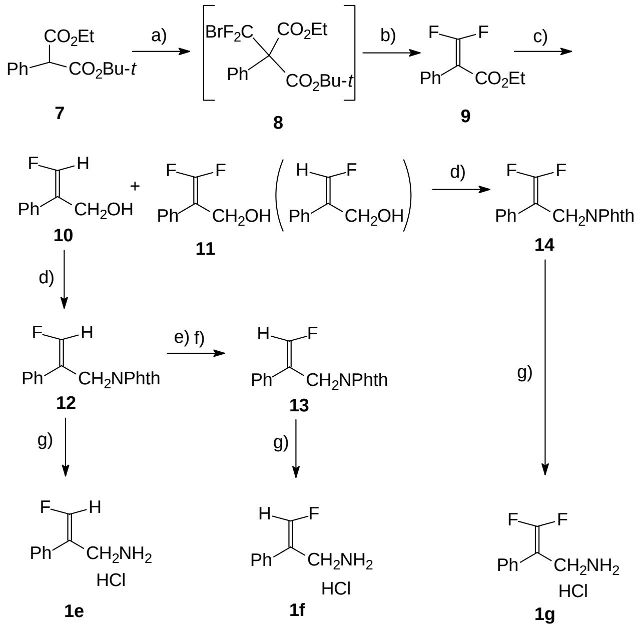
It was reported more than 20 years ago that propargylamine and 2-chloroallylamine could inactivate bovine plasma amine oxidase (BPAO).[9] These same types of derivatives were later designed as potential mechanism-based inhibitors of MAO[10] and/or SSAO.[11] Although mammalian SSAOs include both a membrane-bound and circulating plasma form, the ability of haloallylamines other than 2-chloroallylamine to inactivate BPAO was not studied prior to our report that (*E*)- and (*Z*)-3-chloroallylamine are two of the most potent inactivators of this enzyme.[12] On the basis of the desire to evaluate inhibitory selectivities that can be achieved among various mammalian amine oxidases, we were curious about the potential for inactivation of BPAO of the (*E*)- and (*Z*)-3-halo-2-phenylallylamines that Merrell Dow developed originally for inhibition of MAO.[10] We also considered replacing the 2-phenyl group with 2-methyl group, which would result in different electronic and steric attributes. In this paper, we thus describe the inhibitory activities of haloallylamines **1a**–**l** (Fig. 1) toward BPAO.

2. Results

2.1. Synthesis

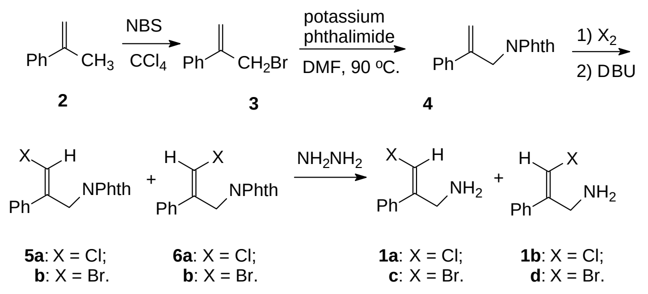
The synthesis of 2-phenylallylamine and compounds **1a**–**c** was carried out as described previously (Scheme 2).[10] Compound **1d** was also obtained by careful silica gel chromatography (eluant petroleum ether–EtOAc 2:1) during the separation of **1c**.

Fluorinated 2-phenylallylamines **1e**–**f** were prepared by a modification of the literature route[10] that was required in our hands to afford sufficient yields (Scheme 3). First, ethyl 2-(*t*-butoxycarbonyl)-2-(bromodifluoromethyl)-phenylacetate (**8**) was obtained by treating ethyl 2-(*t*-butoxycarbonyl)phenylacetate with CBr_2F_2 under basic conditions at room temperature. Heating crude **8** under reduced pressure afforded ethyl 3,3-difluoro-2-phenylacrylate (**9**). Diisobutylaluminum hydride (DIBAL-H) reduction of **9** afforded a 65% yield of (*E*)-2-phenyl-3-fluoroallyl alcohol (**10**) and 15% of 3,3-difluoro-2-phenylallyl alcohol (**11**) that was contaminated with minor inseparable (*Z*)-2-phenyl-3-fluoroallyl alcohol. The Mitsunobu reaction converted alcohols **10** and **11** into the corresponding phthaloyl-protected amines **12** and **14**. Difluorinated **14** was further purified by recrystallization. Isomerization of **12**–**13** was accomplished by a sequence of *syn* bromination followed by NaI-mediated *anti* debromination. Deprotection of **12**, **13**, and **14** with hydrazine and HCl follow-up furnished **1e**, **1f**, and **1g** hydrochloride salts, respectively.

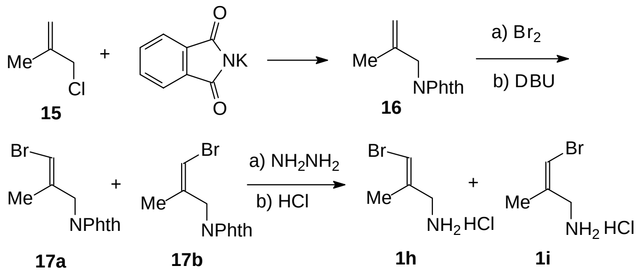


Scheme 3. Reagents: (a) NaH, CBr_2F_2, THF; (b) reduced pressure distillation; (c) DIBAL-H; (d) Ph_3P, DEAD, phthalimide; (e) Br_2; (f) NaI; (g) NH_2NH_2 then HCl.

The preparation of isomeric 3-bromo-2-methylallylamines **1h** and **1i** hydrochloride salts was accomplished as shown in Scheme 4. Treatment of 3-chloro-2-methylpropene with potassium phthalimide in DMF, followed by bromination in CCl_4 and then 1,8-diazobicyclo[5.4.0]undec-7-ene (DBU)-mediated dehydrobromin-



Scheme 2.



Scheme 4.

ation, afforded a mixture of (*E*)- and (*Z*)-1-bromo-2-methyl-3-phthalimido-1-propenes **17a** and **17b**. After hydrazine-mediated deprotection, the mixture of free bases was chromatographed to remove excess hydrazine, and the pooled free base mixture was treated with methanolic HCl to give salts **1h** and **1i**, which were separated by fractional crystallization. The major *E*-isomer **1h** was obtained in pure form, though the minor *Z*-isomer **1i** still contained about 10% of **1h** after repeated crystallization.

The assignment of configuration of the *E*- and *Z*-isomers **1a–d** and **1h**/**1i** was made on the basis of nuclear Overhauser enhancement (NOE) experiments (Fig. 2). Thus, for (*E*)-3-chloro-2-phenylallylamine (**1a**), presaturation of the methylene singlet at 4.05 ppm enhanced the vinyl signal at 6.78 ppm, while the (*Z*)-isomer showed no such enhancement. For these analogs, all (*E*)-isomers exhibited a (+) NOE, while there was no NOE for the (*Z*)-isomers. Once the stereochemistry of the final amine salts was elucidated, the stereochemistry of the phthalimide precursors could be assigned, and we note that elimination of the 1,2-dihalo intermediates always gave mainly the *E*-isomer.

The preparation of **1l** started with reduction of α-chlorocinnamaldehyde to the corresponding known (*Z*)-alcohol **19**,[13] which subsequently was converted to phthalimide derivative **20** by the Mitsunobu reaction (Scheme 5). Deprotection of **20** with hydrazine readily afforded (*Z*)-2-chloro-3-phenylallylamine **1l**. Lastly, the *E* and *Z*-isomers of 3-chloro-3-phenylallylamine (**1j** and **1k**) were prepared starting with the reaction of phenylacetylene with methyl chloromethyl ether in the presence of $ZnCl_2$ as Lewis acid to give a mixture of the known 1,3-dichloro-1-phenylpropene isomers **21a** and **21b**,[14] which were separated by fractional column chromatography. The individual isomers were treated with ammonium hydroxide to give the desired amines **1j** and **1k**, respectively. The configuration at both the chloride and amine stages was established on the basis that the CH_2 *cis* to the phenyl ring in the *E*-isomers is shielded relative to the *trans* CH_2 in the *Z*-isomers.[14]

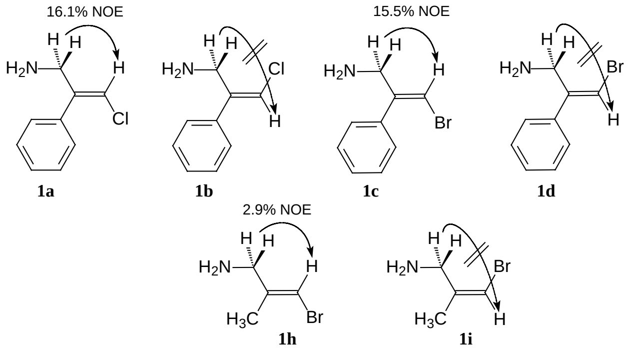
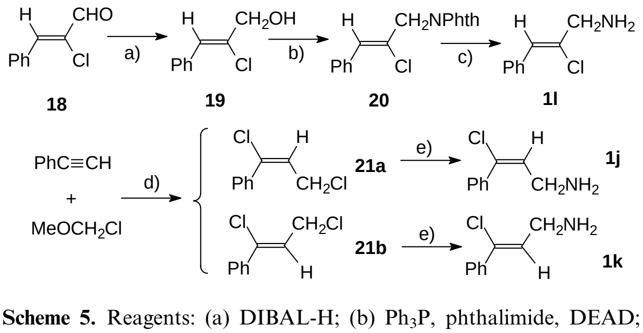


Figure 2. Structural assignments of γ-haloallylamine analogs.



Scheme 5. Reagents: (a) DIBAL-H; (b) Ph_3P, phthalimide, DEAD; (c) NH_2NH_2; (d) $ZnCl_2$; (e) NH_4OH.

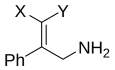
2.2. Inactivation of BPAO by (*E*)- and (*Z*)-3-halo-2-phenylallylamines

Data on the consequence of pre-incubation of 2-phenylallylamine and its various 3-halo analogs with BPAO on its activity are listed in Table 1, alongside data obtained previously for rat brain MAO (**1d** was not tested).[10] In contrast to the weak inhibitory activity seen for the parent compound, the 3-halo derivatives exhibited remarkably potent, time- and concentration-dependent inhibition of BPAO. (*Z*)-3-Fluoro-2-phenylallylamine (**1f**) showed the most potent inhibition, effecting 50% loss of BPAO activity in 40 min at a concentration of 2 μM. The *Z*-isomers of 3-chloro- (**1b**) and 3-bromo-2-phenylallylamine (**1d**) both exhibited efficient, but about 15-fold weaker, inactivations than **1f**. Compared to these three *Z*-isomers, all three *E*-isomers of 3-halo-2-phenylallylamine exhibited approximately 10-fold less effective inhibition, respectively, on the basis of IC_{50} values. Interestingly, the 3,3-difluoro-analog exhibited inhibitory activity in between that of the two configurational 3-monofluoro analogs.

Although the 3-halo-2-phenylallylamines showed pseudo-first order time-dependent inactivation behavior (see Fig. 3 as an example), Kitz and Wilson replots[15] of the data gave lines passing through the origin in every case, suggestive of a lack of saturation and negating the possibility for obtained K_I and k_{inact} parameters. In this situation, the reaction kinetics are best described in terms of the slope of the line passing through the origin, equivalent to k_{inact}/K_I, as if the inactivations represent bimolecular reactions between the enzyme and the inhibitors. However, since the inhibitors have little intrinsic reactivity, this phenomenon is best explained in terms of k_{inact} being fast relative to the dissociation of the initial E–I complex. The relative potencies of inactivation for the 3-halo-2-phenylallylamines are thus best compared kinetically in terms of the k_{inact}/K_I values listed in Table 1. On the basis of these values, it can be seen that the (*Z*)- and (*E*)-3-fluoro analogs are 15–25 times more potent BPAO inhibitors than the corresponding 3-chloro and 3-bromo analogs of the same configuration.

Additional experiments confirmed the irreversibility of BPAO inactivation by **1a–d**, as indicated by re-assay of activity following separation of enzyme from small molecules by gel-filtration. However, 24 h dialysis of the inactivated enzyme preparations was associated with partial recovery of activity (Table 2). Phenylhydrazine titration[16] of the active site after 90% inactivation of

Table 1. The inhibitory potencies of haloallylamine analogs on BPAO and MAO

	X	Y	BPAO inhibition 40 min IC_{50} (μM)	MAO inhibition 15 min IC_{50} (μM)[a]	k_{inact}/K_I (BPAO) ($mM^{-1}\ min^{-1}$)	IC_{50} MAO/IC_{50} BPAO
	H	H	2000	400	n.d.[b]	0.20
1a	Cl	H	311.7	100	0.054	0.32
1b	H	Cl	33.9	500	0.58	15
1c	Br	H	100 (IC_{20})	40	n.d.[b]	
1d	H	Br	29.0		0.52	
1e	F	H	21.4	0.018	0.84	0.0008
1f	H	F	2.0	0.15	13.2	0.075
1g	F	F	8.4	10	2.2	1.2

[a] Data from Ref. 10.

[b] Not determined.

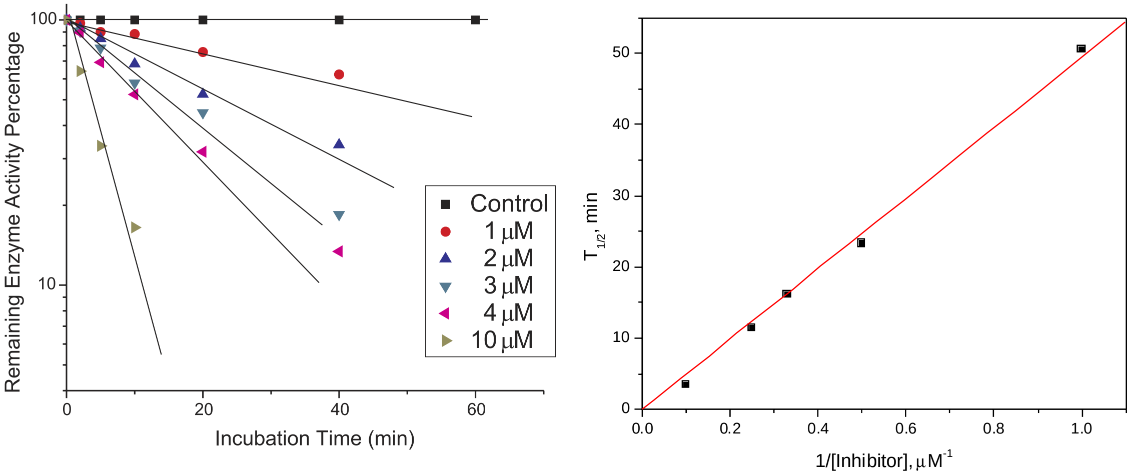


Figure 3. Time-dependent inactivation of BPAO by various concentrations of (*Z*)-3-fluoro-2-phenyl-2-propenamine (**1f**) (left), and Kitz and Wilson replot of the data (right).

Table 2. Reversibility of BPAO inhibition by 3-halo-2-phenylallylamines

Compound	Enzyme activity at end of incubation before gel-filtration[a] (%)	After PDX G. F. 25 (%)	Enzyme activity at end of incubation before dialysis[b] (%)	After dialysis[c] (%)
1a	3.2	6.7	0.9	20
1b	5.2	3.2	2.5	17
1c	5.8	9.1	5.4	31
1d	0	8.7		

[a] **1a** at 1 mM, **1b** at 100 μM, **1c** at 10 mM, and **1d** at 0.5 mM.

[b] **1a** at 1 mM, **1b** at 200 μM, and **1c** at 10 mM.

[c] Enzyme activity after dialysis for 24 h against 50 mM, pH 7.2, sodium phosphate buffer at room temperature.

BPAO by **1a** failed to exhibit the normal development of absorbance at 440 nm due to derivatization of the TPQ cofactor. However, the nitroblue tetrazolium (NBT) assay[17] on the denatured enzyme following inactivation by **1f** was indistinguishable from the denatured control enzyme.

2.3. Inactivation of BPAO by (*E*)- and (*Z*)-3-bromo-2-methylallylamines (1h and 1i)

Since inactivation of BPAO by 3-halo-2-phenylallylamines displayed a general dependence on the relative configuration between the phenyl ring and the halogen, we investigated whether a similar tendency would be observed in the 2-methyl series of inactivators. Both (*E*)- and (*Z*)-3-bromo-2-methylallylamines exhibited very weak inactivation compared to the 2-phenyl inhibitors. In the case of (*Z*)-3-bromo-2-methylallylamine (**1i**, containing 10% **1h**), 50% inactivation was achieved at 1 mM in 150 min, which is a 100-fold higher IC_{50} than the corresponding phenyl analog. The *E*-isomer **1h** exerted somewhat weaker inhibition with an IC_{50} of 1.5 mM at 150 min. The finding that **1h** was a weaker inhibitor suggested that the greater activity of **1i** did not reflect the contaminating **1h**. The small $Z > E$ potency difference here for BPAO inhibition contrasts the 10-fold $Z > E$ potency difference seen for the 2-phenyl series.

Following gel-filtration, a sample of enzyme inactivated by 5 mM **1i** by 97.5% exhibited only slight recovery (to 7%). Upon phenylhydrazine titration of the active site following inactivation by **1i**, the normal development of absorbance at 440 nm was not observed, consistent with **1i** being a mechanism-based inactivator of BPAO.

Despite the weak inhibition for these compounds, time-dependent loss of BPAO activity showed no obvious plateau, suggesting that there was little competing productive turnover and thus, that the weak inhibitory effect principally reflected a weak binding affinity of these compounds. The time-dependent inhibition data for **1h** are shown in Figure 4 (left), and the finding that the Kitz and Wilson replot (Fig. 4, right) intersects the 0–0 origin, again indicates a lack of saturation, so that the inactivation kinetics are best described in terms of $K_{\text{inact}}/K_{\text{m}} = 0.0055\ \text{min}^{-1}\ \text{mM}^{-1}$. The corresponding value for **1i** was $K_{\text{inact}}/K_{\text{m}} = 0.032\ \text{min}^{-1}\ \text{mM}^{-1}$. These values are 2–3 orders of magnitude lower than what was observed for **1a**–**g**. The apparently weak activity and selectivity in the methyl series and presumed difficulty in synthesizing the corresponding fluoro analogs led to a decision not to evaluate additional analogs.

2.4. Inactivation of BPAO by halogenated 3-phenylallylamines (1j–l)

Unlike the behavior of 2-phenylallylamine as a weak inhibitor, 3-phenylallylamine (cinnamylamine) was found to be a mixed substrate-inhibitor of BPAO,[18] exhibiting a maximal turnover rate to cinnamaldehyde (monitored at 285 nm) at 10 µM. Inhibition must in part be mediated at an ancillary site, since competitive inhibition due to substrate activity would not explain the decreased turnover at higher concentrations. Halogenated 3-phenylallylamines behaved as inactivators of BPAO, but the time course showed a different pattern compared to 3-halo-2-phenylallylamines. Thus, after a quick time-dependent loss of enzyme reactivity, an apparent plateau was reached, suggestive of a partitioning between inactivation and turnover to a product with low or nil inhibitory activity (the example for **1j** is shown in Fig. 5). IC_{50} values at the plateau points and partition ratios are listed in Table 3. The large partition ratios suggest that these amines are good substrates of BPAO, though the extent of inactivation achieved at each concentration was shown to be irreversible by observing no increase in enzyme activity after gel-filtration.

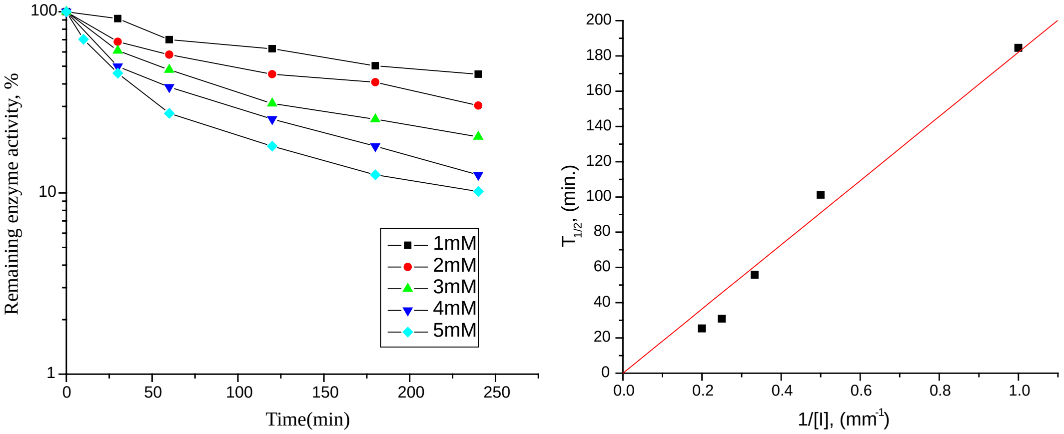


Figure 4. Time-dependent loss of BPAO activity upon incubation with various concentrations of (*E*)-3-bromo-2-methylallylamine (**1h**) (left), and Kitz and Wilson replot of the data (right).

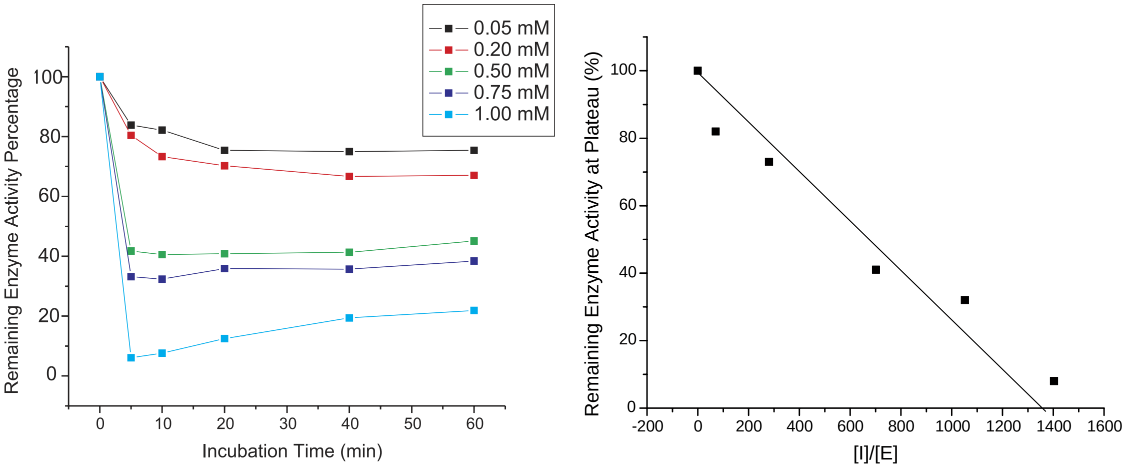


Figure 5. Time-dependent inactivation of BPAO by various concentrations of (*Z*)-3-chloro-3-phenylallylamine **1j** (left) and partition ratio plot.

Table 3. IC_{50} values and partition ratios for the inhibition of BPAO by **1j–l**

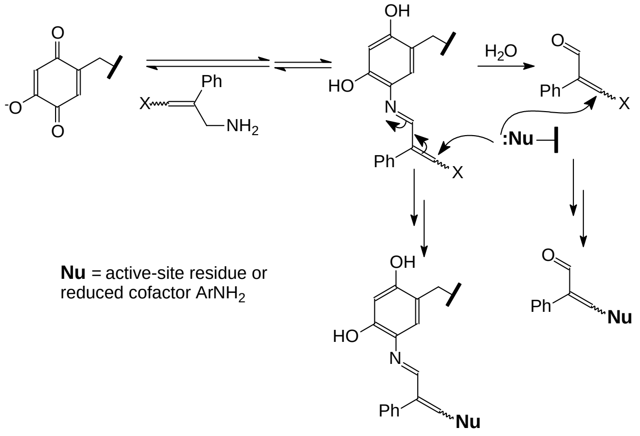
Compound	IC_{50} at plateau (mM)	Partition ratio
1j	0.46	1360
1k	0.40	1280
1l	0.8	2400

3. Discussion and conclusion

In this study, 2-phenyl-3-haloallylamines were found to be potent irreversible inactivators of BPAO. The general order of potency was F ≫ Br ~ Cl, and in each case, the *Z*-isomers were about 10-fold more potent inactivators than the *E*-isomers (the 3,3-difluoro isomer exhibited activity in between the 3-(*Z*)-fluoro and 3-(*E*)-fluoro isomers). As shown in Table 1, this configurational preference is opposite to that seen for inhibition of MAO, where the *E*-isomers are more potent. A priori, there is no reason for inhibition of these two amine oxidases to display the same stereoselectivity, since the respective active sites have no apparent homology, and the mechanisms of oxidative deamination are entirely different. Data on the relative inhibitory activity of this full series of compounds (**1a**–**g**) on SSAO are not available, but for the case of 2-(3,4-dihydroxyphenyl)-3-fluoroallylamine, the *E* configurational isomer was found to be about six times more potent than the *Z*-isomer for the SSAO activity in rat aorta homogenates.[11a] Thus even within the quinone-dependent enzyme class, there may be different stereoselectivities between the tissue-bound and soluble enzymes. Opposite configurational rank orders have important implications on the design of selective inhibitors for the different enzyme families.

Interestingly, in the case of MAO, the 3-fluoro isomers are also more potent than the 3-chloro and 3-bromo analogs, by an even greater extent than in the case of BPAO. Thus, as shown by the limited IC_{50} ratio data that can be extracted (last column in Table 1), although the 3-fluoro analogs are the most potent inhibitors of BPAO, it is the 3-chloro analogs (along with the 3,3-difluoro analog) that are most *selective* for inhibition of BPAO over MAO.

Although the phenylhydrazine and nitroblue tetrazolium (NBT) redox cycling assays were not conducted on the whole series, we assume a common mechanism of inhibitory action for all 3-halo-2-phenylallylamines. Thus, the finding of a lack of phenylhydrazine reactivity with **1a**-inactivated BPAO but retention of cofactor redox activity of the **1f**-inactivated BPAO suggests that these analogs inactivate BPAO by a mechanism that involves covalent alkylation of an active site residue by the electrophilic α,β-unsaturated aldehyde turnover products, in a manner that blocks access of substrate benzylamine and phenylhydrazine to the quinone cofactor. The great enhancement in inhibitory potency of 2-phenylallylamine by addition of the 3-halo substituent leads us to suggest an efficient addition–elimination mechanism of inactivation (Scheme 6), as we proposed in the case of the simple 3-chloroallylamine *E*- and *Z*-isomers.[12] Covalent adduction of an active-site nucleophile could occur at either product Schiff base stage or subsequent to hydrolytic release of α,β-unsaturated aldehyde. Alternatively, the latter aldehyde could 'bite back' and alkylate the reduced cofactor prior to release into bulk solvent, as we have seen in another case.[19] Regardless of the alkylation mode, such mechanism would be consistent with the eventual partial recovery of activity seen upon dialysis, since the adducts shown could eventually hydrolyze (e.g., by a second addition–elimination reaction). It is not clear whether the enhanced potency of the fluoro with respect to bromo and chloro analogs reflects a steric preference or electronic activation effect.



Scheme 6.

Replacement of the 2-phenyl group with a 2-methyl group in the 3-bromo series resulted in substantially reduced inhibitory potency, though the mechanism appears to be the same, as no evidence of a partitioning phenomenon (plateauing of enzyme activity) was observed. Although not directly addressed, we interpret the weaker inhibitory activity to reflect weaker active site binding of these analogs. Weaker inhibition can also be rationalized in that the methyl-substituted haloacrylaldehyde turnover product is a weaker Michael acceptor than the corresponding phenyl analogs and would thus afford a less efficient inactivating covalent binding activity. In the 2-methyl case, there was also a smaller $Z > E$ potency difference.

In contrast to the 2-phenyl and 2-methyl analogs, the 3-phenyl chloroallylamines **1j–l** were moderately potent inactivators that all showed a plateau phenomenon, suggestive of partitioning between productive turnover and inactivation. The (*E*)- and (*Z*)-3-chloro-3-phenyl analogs were more efficient inactivators than the (*Z*)-2-chloro-3-phenyl analog on the basis of an observed 2-fold lower IC_{50} and 2-fold lower partition ratio. Exactly why there should be a different behavior observed for these derivatives in terms of the mechanism proposed in Scheme 6 is not clear, but it is evident that both electronic and steric effects on the postulated addition–elimination process would be different for 2-phenyl versus 3-phenyl substitution. Further work will be required to iron out details of enzyme modification responsible for the various inhibitory activities seen for this series of compounds.

4. Experimental

4.1. General

^{1}H NMR spectra were obtained on a Varian Gemini 300 instrument (^{13}C NMR at 75 MHz) or a Vrian Gemini 200 instrument (^{13}C NMR at 50 MHz) with chemical shifts being referenced to TMS or the solvent peak. In the ^{13}C NMR line listings, attached proton test (APT) designations are given as (+) or (−) following the chemical shift. High-resolution mass spectra (HRMS, FAB) were obtained at 20–40 eV on a Kratos MS-25 A instrument. UV–vis spectra were obtained using a jacketed (temperature-controlled) cell compartment and Perkin-Elmer PECSS software. Bovine plasma amine oxidase was purchased from Sigma or Worthington Biochemical Corporation. The water used for all studies was doubly distilled and deionized. All solvents, reagents, and organic fine chemicals were the most pure available from commercial sources. All column chromatography was conducted using flash grade silica gel using a hexanes–EtOAc gradient (increasing polarity), unless specified otherwise.

4.2. Synthesis

4.2.1. Preparation of 3-halo-2-phenylallylamines (1a–d). Compounds **1a**–**d** were prepared according to McDonald's procedure.[10]

(*E*)-3-Chloro-2-phenylallylamine · HCl (**1a**): ^{1}H NMR (D_2O) δ 4.05 (d, 2H, J = 1 Hz), 6.78 (t, 1H, J = 1 Hz), 7.46–7.56 (5H); ^{13}C NMR (D_2O) δ 47.3, 124.5, 131.9, 132.2, 132.5, 136.6, 138.5.
(*Z*)-3-Chloro-2-phenylallylamine · HCl (**1b**): ^{1}H NMR (D_2O) δ 4.31 (s, 2H), 6.76 (s, 1H), 7.47 (br s, 5H); ^{13}C NMR (D_2O) δ 40.8, 125.5, 129.7, 132.0, 136.0, 137.7, 138.1.
(*E*)-3-Bromo-2-phenylallylamine · HCl (**1c**): ^{1}H NMR (D_2O) δ 3.95 (s, 2H), 6.85 (s, 1H), 7.34–7.46 (5H); ^{13}C NMR (D_2O) δ 48.0, 113.6, 131.4, 131.9, 132.1, 137.8, 140.9.
(*Z*)-3-Bromo-2-phenylallylamine · HCl (**1d**): ^{1}H NMR (D_2O) δ 4.32 (s, 2H), 6.95 (s, 1H), 7.44 (br s, 5H); ^{13}C NMR (D_2O) δ 43.5 (+), 115.4 (−), 129.7 (−), 132.0 (−), 132.1 (−), 138.9 (+), 140.5 (+).

4.2.2. Preparation of ethyl 3,3-difluoro-2-phenylacrylate (9). To a solution of *tert*-butyl phenylacetate (0.58 g, 3 mmol) in THF was added lithium diisopropylamide in THF (1 M, 6 mL) at −78 °C for 30 min. The resulting solution was treated with ethyl chloroformate (0.43 g, 3 mmol) and stirred for 2 h, then at room temperature for 1 h. The THF was removed, and saturated aqueous NH_4Cl (50 mL) was added, and the mixture was extracted with ether (3 × 50 mL). The combined organic layer was dried (Na_2SO_4) and concentrated in vacuo. The residue was distilled to give ethyl 2-(*tert*-butoxycarbonyl)phenylacetate (**7**) in 87% yield: ^{1}H NMR ($CDCl_3$) δ 1.28 (t, 3H, J = 7.1 Hz), 1.46 (s, 9H), 4.21 (q, 1H, J = 7.1 Hz), 4.22 (q, 1H, J = 7.1 Hz), 4.53 (s, 1H), 7.34–7.38 (5H); ^{13}C NMR ($CDCl_3$) δ 14.1, 27.9, 59.1, 61.7, 82.4, 128.1, 128.6, 129.3, 133.3, 167.3, 168.5.

To a solution of **7** (5.2 g, 20 mmol) in THF (80 mL) was suspended NaH (1.6 g, 60% by weight in oil, 40 mmol) at 0 °C. After H_2 was evacuated, dibromodifluoromethane (6.2 g, 60 mmol) was introduced at room temperature. The reaction mixture was stirred for 5 days and then quenched carefully with water (100 mL). The solution was extracted with ethyl acetate (3 × 50 mL), and the combined organic layer was dried (Na_2SO_4) and concentrated in vacuo at room temperature. The residue was distilled at 110 °C under reduced pressure to give pure **9** in 77% yield: bp 55 °C/3 mmHg; ^{1}H NMR ($CDCl_3$) δ 1.31 (t, 3H, J = 7.1 Hz), 4.29 (q, 2H, J = 7.1 Hz), 7.31–7.42 (5H); ^{13}C NMR ($CDCl_3$, partial spectrum) δ 26.4, 73.9, 140.7, 142.26, 142.31, 142.4, 171.9 (dd, J = 309.6 and 296.0 Hz).

4.2.3. DIBAL-H reduction of 9. To a solution of **9** (2.12 g, 10 mmol) in THF (60 mL) at −78 °C was added a solution of DIBAL-H in hexanes (1.5 M, 20 mL, 30 mmol). The reaction mixture was stirred for 1 h and warmed to 0–5 °C for another 2 h. The reaction was quenched by methanol and then with 10% aqueous KOH (10 mL). The organic layer was collected and the aqueous solution was extracted with ether (3 × 20 mL). The combined organic layer was dried (Na_2SO_4) and concentrated in vacuo. The residue was purified by column chromatography to give pure (*E*)-3-fluoro-2-phenyl-2-propen-1-ol (**10**): yield 65%; ^{1}H NMR ($CDCl_3$) δ 4.38 (d, 2H, J = 4.4 Hz), 6.88 (d, 1H, J = 82.5 Hz), 7.33–7.44 (m, 3H), 7.54 (d, 2H, J = 7.4 Hz). A second column fraction, contaminated by inseparable (*Z*)-3-fluoro-2-phenyl-2-propen-1-ol, was 3,3-difluoro-2-phenyl-2-propen-1-ol (**11**): yield 15%; ^{1}H NMR ($CDCl_3$) δ 4.53 (t, 2H, J = 2.5 Hz), 7.32–7.47 (5H).

4.2.4. Preparation of phthaloyl-protected allylamines (12–14). To a solution of 3-fluorinated-2-phenylallyl alcohol **10** or **11** (5 mmol), Ph_3P (1.78 g, 7.5 mmol), and phthalimide (1.1 g, 7.5 mmol) in THF (60 mL) was added diethyl azodicarboxylate (DEAD) (1.3 g, 7.5 mmol) at 0 °C. After addition, the mixture was warmed to room temperature and reacted overnight. The solvent was removed and the residue was purified by column chromatography. From **10** was obtained **12** in 82% yield: ^{1}H NMR ($CDCl_3$) δ 4.57 (dd, 2H, J = 3.5 and 1.1 Hz), 6.82 (0.5H, one peak of ^{19}F-coupled vinyl doublet), 7.23–7.37 (3H + 0.5H of ^{19}F-coupled vinyl doublet), 7.51 (m, 2H), 7.65 (m, 2H), 7.77 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 38.0 (d, J = 10 Hz), 118.2, 123.4, 128.1, 128.4, 128.5, 128.6, 131.9, 132.0, 133.0, 134.1, 148.4 (d, J = 266.2 Hz), 167.9. From impure **11** was obtained, after column chromatography, crude **14**, which was further purified by recrystallization: yield 62%; ^{1}H NMR ($CDCl_3$) δ 4.70 (t, 2H, J = 0.6 Hz), 7.22–7.38 (5H), 7.64–7.79 (4H); ^{13}C NMR ($CDCl_3$) δ 35.5 (m), 123.4, 128.0, 128.6, 128.7, 128.8, 131.8, 134.1, 154.7, 167.7.

To a solution of **12** (0.56 g, 2 mmol) in CH_2Cl_2 (10 mL) at 0 °C was added bromine (0.35 g, 2.2 mmol) in CH_2Cl_2 (5 mL). The cooling bath was then removed and the solution was stirred for 24 h. After removal of organic solvent and excess Br_2 in vacuo, NaI (15 g, 100 mmol) and acetone (100 mL) were added, and the solution was heated at reflux for 4 h. The reaction mixture was decolorized by 10% aqueous Na_2SO_3 (80 mL). Acetone was removed, and the water layer was extracted with ether (3 × 80 mL). The combined ether extract was dried (Na_2SO_4) and concentrated in vacuo. Column chromatography of the residue gave pure starting material **12** and its *Z*-isomer **13** (0.21 g, 38%): ^{1}H NMR ($CDCl_3$) δ 4.83 (dd, 2H, $J = 2.6$ and 1.3 Hz), 6.86 (d, 1H, $J = 82.8$ Hz), 7.24–7.33 (5H), 7.64 (m, 2H), 7.75 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 34.2 (d, $J = 7.0$ Hz), 120.3 (d, $J = 5.8$ Hz), 123.3, 127.4, 127.5, 128.2, 128.7, 131.9, 133.5, 134.0, 146.0 (d, $J = 265.7$ Hz), 167.8.

4.2.5. Preparation of 3-fluorinated-2-phenylallylamines (1e–g). A mixture of phthaloyl-protected fluorinated amine (1 mmol) and hydrazine hydrate (100 mg, 2 mmol) in CH_3OH (10 mL) was refluxed for 3 h. Aqueous HCl (6 N, 1 mL) was added and refluxing was continued for 30 min. The mixture was cooled and filtered to remove phthalhydrazide. Evaporation of the filtrate left a solid residue which typically contained traces of hydrazine hydrochloride even after recrystallization. Thus, the weighed residue was dissolved in aqueous THF containing 4 equiv of Na_2CO_3, to which was added di-*tert*-butyl dicarbonate. After 30 min stirring, and TLC showed complete conversion of the free amine to the Boc-protected compound, the solution was concentrated in vacuo to remove the THF and extracted with EtOAc. The organic layer was dried (Na_2SO_4) and evaporated, and the residue was purified by column chromatography. The Boc-protected compound was dissolved in 6 N HCl, and the solution was evaporated to dryness. Excess HCl was removed by repetitive dissolution in water and evaporation. The final residue was recrystallized from EtOH/Et_2O to afford hydrazine-free **1e–g** hydrochloride salts.

Compound **1e** · HCl: yield, 62%; mp, 193–195 °C; ^{1}H NMR (CD_3OD) δ 3.95 (dd, 2H, $J = 3.4$, 0.8 Hz), 6.99 (t, 0.5H, $J = 0.8$ Hz), 7.39–7.54 (5.5H); ^{13}C NMR δ 40.5 (d, $J = 10.6$ Hz), 118.5 (d, $J = 6.4$ Hz), 129.6 (d, $J = 4.0$ Hz), 129.9, 130.0, 132.3, 150.6 (d, $J = 268.6$ Hz). Compound **1f** · HCl: yield, 68%, mp, 156–157 °C; ^{1}H NMR (CD_3OD) δ 4.14 (d, 2H, $J = 2.4$ Hz), 7.02 (s, 0.5H), 7.42–7.49 (5.5H); ^{13}C NMR (CD_3OD) δ 36.0 (d, $J = 7.6$ Hz), 119.8 (d, $J = 6.6$ Hz), 128.2 (d, $J = 3.1$ Hz), 130.0, 130.4, 133.7 (d, $J = 7.0$ Hz), 151.5 (d, $J = 267.0$ Hz).
Compound **1g** · HCl: yield, 42%, mp, 149–150 °C; ^{1}H NMR (CD_3OD) δ 4.02 (t, 2H, $J = 1.9$ Hz), 7.43–7.45 (5H).

4.2.6. Preparation of 2-methyl-3-phthalimido-1-propene (16). To a solution of 3-chloro-2-methyl-1-propene **15** (2 g, 22 mmol) in dry DMF (50 mL) was added potassium phthalimide (3.7 g, 20 mmol). After heating at reflux for 12 h under nitrogen, the reaction mixture was cooled to room temperature and diluted with ice water, at which point the desired product precipitated out. The solid was filtered, washed several times with water and methanol, and recrystallized from hexane and $CHCl_3$ to give **16** in 79% yield: ^{1}H NMR ($CDCl_3$) δ 1.78 (s, 3H), 4.22 (s, 2H), 4.85 (m, 2H), 7.70–7.89 (4H); ^{13}C NMR ($CDCl_3$) δ 20.5, 43.3, 112.0, 123.4, 132.1, 139.4, 168.1.

4.3. Preparation of (*E*)- and (*Z*)-1-bromo-2-methyl-3-phthalimidopropenes (17a and 17b)

To a solution of 2-methyl-3-phthalimido-1-propene **16** (5 g, 15 mmol) in chloroform (50 mL) at −10 °C was added 1.1 equiv of Br_2 dropwise over 10 min. The reaction mixture was stirred for an additional 15 min, and the excess Br_2 was removed by bubbling air for 2 h. Saturated aqueous NaCl was added at 0 °C along with a small amount of pentane (or hexane). The separated organic layer was washed with 2% aqueous $NaHCO_3$ until the pH of the water layer reached ca. 8, dried over Na_2SO_4, and concentrated. The solid was recrystallized from $CHCl_3$ to give 1,2-dibromo-2-methyl-3-phthalimidopropane (1.8 g, 33%): ^{1}H NMR ($CDCl_3$) δ 1.89 (s, 3H), 3.86 (s, 2H), 4.16 (s, 2H), 7.75–7.87 (4H); ^{13}C NMR ($CDCl_3$) δ 29.4 (−), 42.3 (+), 47.5 (+), 46.2 (+), 123.7 (−), 131.8 (+), 134.4 (−), 168.1 (+).

To a solution of 1,2-dibromo-2-methyl-3-phthalimidopropane (1 g, 2.78 mmol) in DMSO (40 mL) was added 1.5 equiv of DBU. The reaction mixture was heated at 90 °C for 10 min followed by further heating at 150 °C for 2 h. The cooled reaction mixture was diluted with ice water and partitioned between EtOAc and water. The organic layer was dried (Na_2SO_4) and concentrated, and the residue was subjected to column chromatography (eluant petroleum ether–EtOAc 2:1) to give 0.5 g (64%) a mixture of **17a/17b**. Compound **17a** (major isomer): ^{1}H NMR ($CDCl_3$) δ 1.85 (s, 3H), 4.28 (s, 2H), 6.28 (s, 1H), 7.70–7.85 (4H); ^{13}C NMR ($CDCl_3$) δ 17.9, 43.3, 106.7, 123.5, 131.9, 134.2, 136.1, 167.9. Compound **17b** (minor isomer): ^{1}H NMR ($CDCl_3$) δ 1.72 (s, 3H), 4.50 (s, 2H), 6.10 (s, 1H), 7.70–7.85 (4H).

4.4. Preparation of (*E*)- and (*Z*)-3-bromo-2-methylallylamine hydrochloride salts (1h and 1i)

To a solution of the **17a/17b** mixture (5 g, 18 mmol) in EtOH (70 mL) was added 1.1 equiv of 98% hydrazine under argon. The mixture was heated at reflux for 3 h at which time the clear solution became almost completely solidified and then was cooled to room temperature. The phthalhydrazide was filtered off and washed with EtOH, and the filtrate was concentrated. The residue was subjected to column chromatography (eluant EtOAc–MeOH containing 1% NH_4OH) to remove NH_2NH_2. The pooled mixture of **1h** and **1i** was concentrated, and the residue was added to a solution of 2 mL of 12 N HCl in MeOH (15 mL) with stirring at room temperature for 1 h. The mixture was concentrated and the last traces of water were removed by azeotropic distillation with toluene. Residual hydrazine hydrochloride was eliminated by recrystallizing the mixture two times from EtOH and once in water, with discarding of the first small amount of solid that crystallized in each case. The remaining liquid was concentrated to give an isomeric mixture of HCl salts, which was recrystallized from acetone–chloroform. The first crop forming within 2 h gave 100% pure (*E*)-isomer **1h** (1.7 g, 49% yield). The residual mother liquid was concentrated under reduced pressure, and the residue was recrystallized from EtOH and diethyl ether. After several times recrystallization, the (*Z*)-isomer **1i** (0.3 g, 8.6%) was obtained, but was still contaminated with 10% of the (*E*)-isomer.

Compound **1h** · HCl: ^{1}H NMR (D_2O) δ 1.82 (s, 3H), 3.59 (s, 2H), 6.45 (s, 1H); ^{13}C NMR (D_2O) δ 19.9, 47.6, 112.1, 136.8; HRMS (FAB) calcd for C_4H_8BrN: (MH^+) *m/z* 149.9918. Found: 149.9912.
Compound **1i** · HCl: ^{1}H NMR (D_2O) δ 1.90 (s, 3H), 3.81 (s, 2H), 6.41 (s, 1H); ^{13}C NMR (D_2O) δ 19.9, 47.5, 111.9, 136.7.

4.4.1. Preparation of (*Z*)-2-chloro-3-phenylallylamine (1l). To a solution of α-chlorocinnamaldehyde **18** (4.2 g, 25 mmol) in CH_2Cl_2 (30 mL) under argon was introduced DIBAL-H (1.5 M in toluene, 21 mL, 31.5 mmol) at −78 °C. The resulting mixture was stirred for 1 h and then warmed to room temperature and quenched by slow addition of water. The precipitate was filtered off and washed with CH_2Cl_2. The filtrate was extracted with CH_2Cl_2 and the combined organic phase was dried (Na_2SO_4) and evaporated to afford the known (*Z*)-2-chlorocinnamyl alcohol **19**[13] as a colorless oil (4.05 g, 96%), which was sufficiently pure for the next step: ^{1}H NMR ($CDCl_3$) δ 4.34 (s, 2H), 6.80 (s, 1H), 7.31–7.42 (3H), 7.64 (d, 2H, *J* = 7.5 Hz); ^{13}C NMR ($CDCl_3$) δ 67.8, 124.9, 128.1, 128.3, 129.2, 132.5, 134.2.

A solution of DEAD (4.2 g, 24 mmol) in THF (25 mL) was added to a mixture of **19** (4.0 g, 23.8 mmol), phthalimide (3.6 g, 24 mmol), and Ph_3P (6.3 g, 24 mmol) in THF (25 mL) at room temperature. The resulting suspension was stirred for 12 h and then poured into ice water (300 mL) under stirring. The white solid that precipitated out was collected by filtration, washed with water, and dried at 80 °C to give pure *N*-(2-chlorocinnamyl)phthalimide **20** (6.52 g, 91%): mp 87–88 °C; ^{1}H NMR ($CDCl_3$) δ 4.63 (d, 2H, *J* = 0.8 Hz), 6.82 (s, 1H), 7.25–7.40 (3H), 7.63 (m, 2H), 7.75 (m, 2H), 7.90 (m, 2H); ^{13}C NMR (APT, $CDCl_3$) δ 45.6 (+), 123.6 (–), 127.3 (+), 127.8 (–), 128.2 (–), 128.3 (–), 129.3 (–), 131.9 (+), 133.9 (+), 134.3 (–), 167.7 (+).

Heating a mixture of **20** (3.0 g, 10 mmol) and hydrazine (85%, 0.75 g, 15 mmol) in ethanol (75 mL) for 1 h, addition of a second aliquot of 15 mmol hydrazine, and continued heating for 1 h, TLC showed that deprotection was complete. The mixture was cooled to room temperature, the solidified phthalhydrazide was removed, the filtrate was concentrated, and the residual oil was dissolved in ether. Ethanolic HCl was added, and the white precipitate (1.4 g, 68%) was collected and purified by recrystallization from EtOH.

Compound **1l** · HCl: mp 237–238 °C; ^{1}H NMR (CD_3OD) δ 4.01 (s, 2H), 7.11 (s, 1H), 7.35–7.45 (3H), 7.70 (m, 2H); ^{13}C NMR (CD_3OD) δ 48.6, 125.6, 129.5, 130.1, 130.5, 132.5, 134.8; HRMS (FAB) calcd for $C_9H_{11}ClN$: (MH^+) *m/z* 168.0580. Found: 168.0579.

4.4.2. Preparation of (*E*)- and (*Z*)-1,3-dichloro-1-phenyl-1-propenes (21a and 21b). To a solution of phenylacetylene (2.10 g, 20 mmol) and methyl chloromethyl ether (2.4 g, 30 mmol) in CH_2Cl_2 (20 mL) was added $ZnCl_2$ (0.68 g, 5 mmol) at room temperature and the resulting suspension was stirred for 30 min. When TLC showed the disappearance of starting material, the reaction mixture was poured into ice-water (300 mL) with stirring and extracted with CH_2Cl_2. The organic layer was collected, washed with 5% aqueous sodium carbonate, dried (Na_2SO_4), and evaporated, and the residue was subjected to fractional column chromatography (eluant hexanes) to give the individual isomeric chlorides. **21a** (*E*-isomer): yield 11%; ^{1}H NMR ($CDCl_3$) δ 4.05 (d, 2H, *J* = 8.5 Hz), 6.20 (t, 1H, *J* = 8.5 Hz), 7.35–7.50 (m, 5H); ^{13}C NMR ($CDCl_3$) δ 41.3 (+), 124.8 (–), 128.5 (–), 128.6 (–), 129.6 (–), 135.7 (+), 137.2 (+). **21b** (*Z*-isomer): yield 12%; ^{1}H NMR ($CDCl_3$) δ 4.42 (d, 2H, *J* = 7.5 Hz), 6.33 (t, 1H, *J* = 8.5 Hz), 7.35–7.45 (m, 3H), 7.63 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 40.8 (+), 122.5 (–), 126.7 (–), 128.5 (–), 129.5 (–), 136.9 (+), 137.3 (+).

4.4.3. Preparation of (*E*)- and (*Z*)-3-chloro-3-phenylallylamines (1j and 1k). Compound **21a** or **21b** (0.19 g, 1 mmol) was dissolved in methanol (5 mL) and added to NH_4OH (100 mL). The resulting mixture was stirred at room temperature overnight, and TLC showed that the reaction was complete. After removal of water and methanol in vacuo, the resulting white solid was dissolved in aqueous Na_2CO_3, and the solution was evaporated to dryness to give a white solid, which was suspended in EtOAc. The NaCl was filtered off, and the filtrate was acidified by addition of ethanolic HCl to give white precipitate, which was collected and washed with ethyl acetate/isopropanol (80/20) to give pure products.

Compound **1j** · HCl: yield 87%; mp 192–193 °C; ^{1}H NMR (CD_3OD) δ 3.62 (d, 2H, *J* = 7.5 Hz), 6.11 (t, 1H, *J* = 7.5 Hz), 7.40–7.50 (5H); ^{13}C NMR (APT, CD_3OD) δ 39.4 (+), 122.1 (–), 129.7 (–), 129.9 (–), 131.0 (–), 136.9 (+), 139.4 (+); HRMS (FAB) calcd for $C_9H_{11}ClN$: (MH^+) *m/z* 168.0580. Found: 168.0575.
Compound **1k** · HCl: yield 83%; mp 187–188 °C; ^{1}H NMR (CD_3OD) δ 3.93 (d, 2H, *J* = 7.0 Hz), 6.37 (t, 1H, *J* = 7.0 Hz), 7.41–7.44 (3H), 7.68 (m, 2H); ^{13}C NMR (APT, CD_3OD) δ 39.4 (+), 119.5 (–), 127.7 (–), 129.8 (–), 130.9 (–), 130.9 (–, 137.9 (+), 139.8 (+); HRMS (FAB) calcd for $C_9H_{11}ClN$: (MH^+) *m/z* 168.0580. Found: 168.0577.

4.5. Time-dependent inactivation of BPAO by candidate inhibitors

A 0.9 mL aliquot of a solution of candidate inhibitor in 100 mM potassium phosphate buffer, pH 7.2, was mixed with BPAO (0.1 mL, about 8 μM) and incubated at 30 °C aerobically. Aliquots (0.1 mL) were periodically withdrawn using disposable calibrated Drummond micropipettes and diluted with 1.0 mL of benzylamine (5 mM in 50 mM sodium phosphate buffer, pH 7.2) in a 1 cm quartz cuvette (1.5 mL volume). The rate of oxidation of benzylamine to benzylaldehyde was measured by recording the increase in absorbance at 250 nm for

1 min and compared to the rate of benzylamine oxidation in a companion control solution of enzyme without inhibitor. The concentration of active BPAO was estimated from the rate of benzylamine oxidation as described previously.[20]

4.6. Irreversibility of BPAO inhibition

(*a*) *By gel-filtration.* The irreversibility of the inhibition was checked by applying enzyme preparations (0.5 mL) which were 90–95% inhibited to a PDX G. F. 25 column (1 × 7.8 cm), equilibrated with 100 mM sodium phosphate buffer (pH 7.2), to separate non-covalently bound small molecules. Control runs established that no enzyme elutes in the first 33 drops, and that in the subsequent 10 aliquots of 3 drops each, all enzyme elutes in aliquots 3–7. Percent activity following gel-filtration was determined comparing the activity between two gel-filtrated incubations of enzyme in the absence and presence of inhibitor. The same initial protocol had to be worked out each time the column was changed. (*b*) *By dialysis.* The irreversibility of the inhibition was also checked by adding 0.5 mL enzyme preparations which were 90–95% inhibited to dialysis tubing (6.4 mm Spectrum Spectro/Pro membrance, MW cutoff 12,000–14,000), following by dialysis against 100 mM sodium phosphate buffer, pH 7.2, at room temperature for periods up to 24 h. Percent activity was determined by comparing the enzyme activity between two dialysis experiments, following incubation of enzyme in the absence and presence of inhibitor.

4.7. Phenylhydrazine titration of active site after inactivation of BPAO with inactivators

Incubation of the enzyme with the test inactivator in 100 mM sodium phosphate buffer, pH 7.2, was allowed to proceed at 30 °C until there was more than 80% loss of activity after gel-filtration or dialysis. A control was run at the same time. To the gel filtered or dialyzed enzyme fractions was added 4 μL of phenylhydrazine hydrochloride (2 mM) in water and the absorbance at 440 nm was measured. The percentage decrease in absorbance at 440 nm was determined compared to the control experiment.

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Supplementary material

Plots of time-dependent inhibition of BPAO by **1a**–**e**,**g**,**i**,**k**,**l**, including Kitz–Wilson replots for inhibitors lacking plateau behavior and partition ratio plots for inhibitors exhibiting plateau behavior. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2005.09.065.

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