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Highly potent 3-pyrroline mechanism-based inhibitors of bovine plasma amine oxidase and mass spectrometric confirmation of cofactor derivatization

Yuming Zhang, Chongzhao Ran, Guangyin Zhou and Lawrence M. Sayre*

Department of Chemistry, Case Western Reserve University, Cleveland, OH 44106, USA

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Abstract—Despite the quinone-dependent copper amine oxidases being described as having the ability to metabolize unbranched primary amines to the corresponding aldehydes, we previously showed that the secondary amines 3-pyrrolines are metabolized as mechanism-based inactivators of bovine plasma amine oxidase (BPAO), and that the 3-(3-nitro-4-methoxyphenyl)-substituted analog was a particularly potent and efficient inactivator. We now show that additional 3-aryl-3-pyrrolines containing highly electron-withdrawing aryl groups (pyridyl, quinolyl, isoquinolyl, and pentafluorophenyl) are some of the most potent inactivators of BPAO reported to date. We also provide mass spectroscopic confirmation of the proposed mechanism of inhibition involving pyrrolylation of the active-site cofactor, through identification by MALDI-TOF and LC–ESI-MS/MS of the (3-arylpyrrol-1-yl)resorcinol derivatives of the cofactor-containing thermolytic peptides.

1. Introduction

The copper-containing amine oxidases are typically defined in terms of their ability to convert unbranched primary amines to the corresponding aldehydes, through the utilization of an active-site 2,4,5-trihydroxyphenylalanine quinone (TPQ)-dependent transamination mechanism.[1,2] Interest in the selective inhibition of these enzymes has increased in recent years with the realization of their important physiological roles in plants,[3,4] and particularly with the recognition that the human enzymes in this class, known as semicarbazide-sensitive amine oxidases, appear to be involved in a spectrum of metabolic and signaling pathways.[5–8]

Despite the historical characterization of TPQ-dependent enzymes as being capable of metabolizing only primary amines, a factor that distinguishes them from their flavin-dependent cousins, we recently showed that 3-pyrrolines, though being secondary amines, are metabolized by bovine plasma amine oxidase (BPAO) as mechanism-based inactivators.[9,10] We proposed, with the support of optical spectroscopic and model studies, a transamination of the cofactor-derived iminium substrate Schiff base and subsequent irreversible aromatization, resulting in a stable pyrroloresorcinol cofactor derivative (Scheme 1) that had no tendency to be reoxidized.[9,10] We found that 3-aryl-3-pyrrolines are particularly efficient irreversible inactivators of BPAO, that electron-withdrawing aryl substituents increase inactivation potency, and that the parent compound 3-phenyl-3-pyrroline is a pure substrate for the flavin-dependent mitochondrial monoamine oxidase B from bovine liver.[10]

On the basis that 3-(4-methoxy-3-nitrophenyl)-3-pyrroline exhibited an IC_{50} of 15 μM at 3 min of incubation,[10] we envisioned that additional analogs bearing electron-withdrawing aryl substituents might exhibit even more pronounced inactivation potency. We now report on several new 3-aryl-3-pyrrolines, where the 3-aryl group is pentafluorophenyl, pyridyl, quinolyl, or isoquinolyl, some of which are among the most potent inhibitors of BPAO that have been reported to date. This is remarkable considering that these compounds are secondary amines. We also report mass spectroscopic studies for two analogs that confirm modification of the active site TPQ cofactor in the inactivated enzyme.

Keywords: Copper amine oxidase; Bovine plasma amine oxidase; Mechanism-based inhibitor; Enzyme inactivator; 3-Pyrroline.
* Corresponding author. Tel.: +1 216 368 3704; fax: +1 216 368 3006; e-mail: LMS3@case.edu
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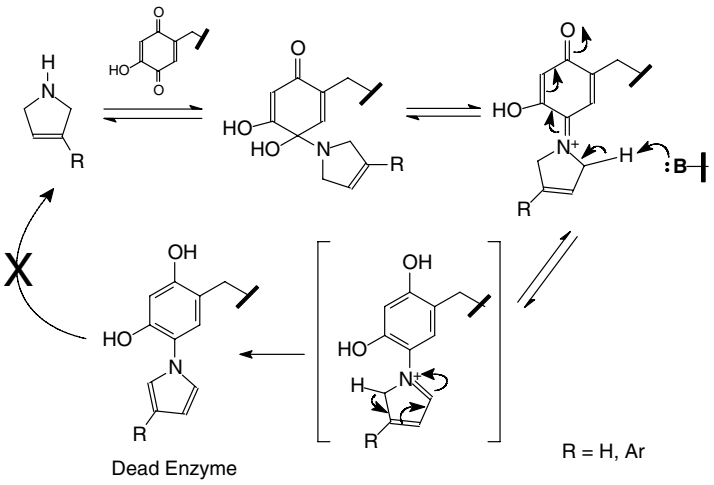
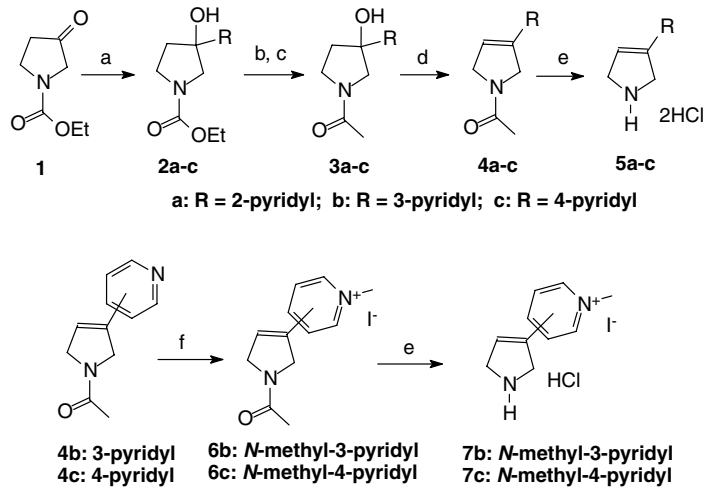


Scheme 1. Mechanism of copper amine oxidase inactivation by 3-pyrrolines.



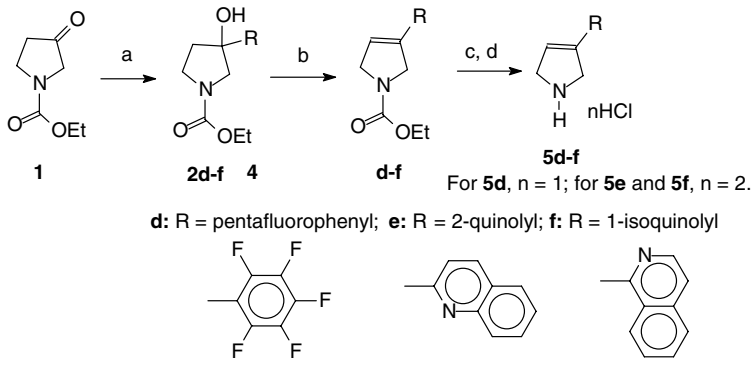
Scheme 2. Synthesis of 3-pyridyl-3-pyrrolines **5a**–**c** and two corresponding *N*-methylpyridinium derivatives **7b** and **7c**. Reagents and conditions: (a) for **2a** and **2b**: 2- or 3- bromopyridine, *n*-BuLi, ether, −78 °C, then **1**; for **2c**: 4-bromopyridine, THF, *n*-BuLi, −78 °C, then **1**; (b) MeOH/KOH/H_2O, reflux; (c) pH 10–12, Ac_2O, 25 °C; (d) $SOCl_2$, 25 °C; (e) EtOH/12 N aqueous HCl (1:1 v/v), reflux; (f) excess CH_3I, CH_2Cl_2, 25 °C.

2. Results and discussion

2.1. Chemistry

Previous preparations of 3-aryl-3-pyrrolines were based on the reaction of aryl Grignard reagent with ethyl 3-oxopyrrolidine-1-carboxylate (**1**). However, in the case of pyridyl reagents, initial experiments revealed that the corresponding Grignard reagents reacted inefficiently with **1**. By lithium–halogen exchange, the more reactive pyridyllithium reagents were generated. Thus, reaction of 2-, 3-, or 4-bromopyridine with *n*-butyllithium in THF at −78 °C,[11] and reaction of the resulting pyridyllithiums with **1** gave the isomeric ethyl 3-(2-, 3-, or 4-pyridyl)-3-hydroxypyrrolidine-1-carboxylates **2a**–**c** (Scheme 2). For synthesis of **2a** and **2b**, higher yields (50–70%) were obtained using ether rather than THF (15–20% yields) as the reaction solvent. For **2c**, because of the low solubility of 4-pyridyllithium in ether, THF was employed to give the product in moderate yield. Attempted conversion of **2a**–**c** to the desired 3-pyrrolines by deprotection (aqueous methanolic KOH) and subsequent strong acid-mediated dehydration as described for the preparation of simple 3-arylpyrrolines failed in these cases (a tarry product mixture was obtained). This failure probably reflects the strong electron-withdrawing property of the protonated pyridine ring, that destabilizes the E1 carbocationic intermediate.

Resorting to thionyl chloride to achieve dehydration,[12] we feared that subsequent base-mediated deprotection of the carbamate would result in isomerization of the C=C. We thus chose to convert the *N*-protecting group to *N*-acetyl prior to dehydration, so that we could use an acid deprotection step following $SOCl_2$-mediated dehydration. Thus, deprotection of **2a**–**c** and reprotection with Ac_2O followed by $SOCl_2$-mediated dehydration afforded *N*-acetyl 3-pyrrolines **4a**–**c** along with variable amounts of 3-chloro-3-pyridyl and 2-pyrroline side products. Isolated compounds **4a**–**c** were converted to



Scheme 3. Synthesis of 3-aryl-3-pyrrolines **5d-f**. Reagents and conditions: (a) arylbromide, *n*-BuLi, ether, −78 °C, then **1**; (b) $SOCl_2$, 25 °C (c) 48% HBr, AcOH, reflux; (d) 12 N HCl, 25 °C.

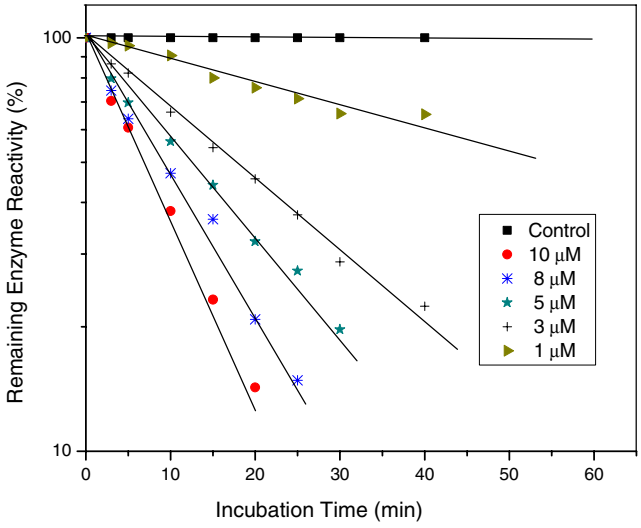


Figure 1. Time-dependent inactivation of BPAO (1 μM) by various concentrations of 3-(2-quinolyl)-3-pyrroline (**5e**) in pH 7.2 sodium phosphate buffer at 30 °C.

the dihydrochloride salts of the target 3-pyridyl-3-pyrrolines (**5a**–**c**·2HCl) using 6 N HCl in 50% aqueous ethanol. To further enhance the electron-withdrawing property of the 3-aryl substituent, pyridinium salts were made by methylation of the *N*-acetyl-protected pyrrolines **4b**, **c** with MeI prior to *N*-deprotection, affording pyrrolines **7b**, **c** following hydrolysis. However, **4a** failed to undergo methylation, probably due to steric hindrance.

We later found that the *N*-carbamoyl group could be removed under non-basic conditions using a mixture of aqueous 48% HBr and acetic acid, which does not affect the 3-pyrroline C=C. Thus, additional 3-pyrrolines, bearing the electron-withdrawing pentafluorophenyl, 2-quinolyl, and 1-isoquinolyl as the 3-aryl substituent, were prepared using the more expedient route shown in Scheme 3, where $SOCl_2$-mediated dehydration was carried out directly on alcohols **2d**–**f**. Although the ethyl carbamate could not be deprotected with 12 N HCl, following deprotection with HBr, the resulting HBr salts of **5d**–**f** salts were transformed to the HCl salts of **5d**–**f** by co-evaporating three times with an excess of aqueous HCl.

2.2. Evaluation of inhibitory potency

The time-dependent inhibition of BPAO by the new 3-aryl-3-pyrrolines was determined by pre-incubating approximately 1 μM BPAO with varying concentrations of the inhibitors at 30 °C, pH 7.2. The enzyme activity in aliquots taken over time was determined by the standard benzylamine assay, monitoring the rate of formation of benzaldehyde at 250 nm. As with the example shown (**5e**, Fig. 1, remaining in Supplementary data), plots of log activity versus time indicated strictly pseudo first-order loss of activity without any plateau being apparent even at low concentrations of inhibitor. Thus, IC_{50} values are time-dependent, but a sense of the relative potency of the various 3-aryl-3-pyrroline analogs relative to the parent 3-phenyl compound and previously reported[10] analogs **8** and **9** (Chart 1) can be gained by comparing the concentrations of inhibitors needed to produce 50% inactivation at a fixed time of 20 min (Table 1). In addition, Kitz and Wilson replots[13] of inactivation half-life data as a function of concentration were constructed in an effort to obtain kinetic constants k_{inact} and K_i. For the weaker 4-pyridyl-substituted inhibitor **5c** (activity vs. time and Kitz and Wilson plots are in Supplementary data), kinetic parameters could be determined. However, for the more potent inhibitors, the Kitz and Wilson plots passed so close to the 0–0 origin (Fig. 2 shows an example for **5e**) that we could not ensure a statistically significant non-zero intercept. This behavior was seen for the previously described potent 3-aryl-3-pyrroline inhibitors,[9,10] where we suggested that the *apparent* lack of saturation presumably reflected rapid conversion to inactivated enzyme of the enzyme–substrate complex, relative to its dissociation. In these cases it is deemed best not to attempt to extract the individual kinetic constants, and the relative potencies of the analogs are thus instead best compared in terms of the slopes of the Kitz–Wilson plots, which formally represent the bimolecular rate constants k_{inact}/K_i. The overall rank order of potency within the series **5a**–**5f** is **5a** > **5f** > **5b** > **5e** > **5d** > **5c**, although some of the differences are small. However, since the electron-withdrawing effects of 2- and 4-pyridyl should be similar, the substantial difference in potency between **5a** and **5c** suggests that the electronic effect is not the only factor governing inhibitory efficiency. Most importantly, com-

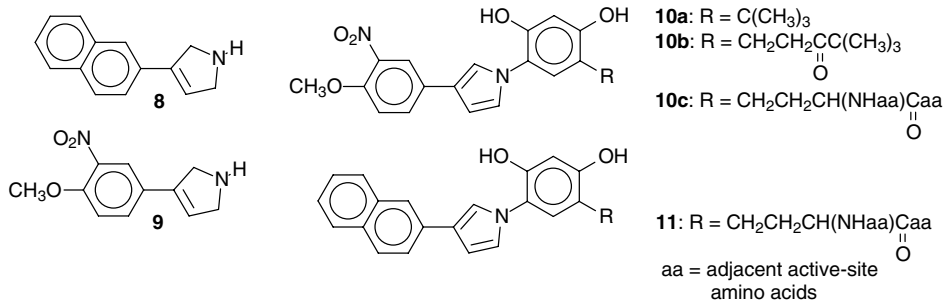


Chart 1.

Table 1. Inactivation of BPAO by 3-substituted-3-pyrrolines (**5a**–**f**, **7b**–**c**, **8**, **9**) at 30 °C

Inhibitors	20 min IC_{50} (μM)	Kinetic data k_{inact}/K_i ($mM^{-1}min^{-1}$)
3-Phenyl-3-pyrroline[a]	160	0.23
8[b]	70	0.53
9[b]	4.4	7.0
5a	0.75	65.
5b	1.5	24.
5c	50	1.2 (K_i = 0.076 mM, k_{inact} = 0.091 min^{-1})
5d	6.1	6.0
5e	2.7	13.
5f	0.86	40.
7b	500	
7c	>1000	

[a] Data from Ref. 9.
[b] Data from Ref. 10.

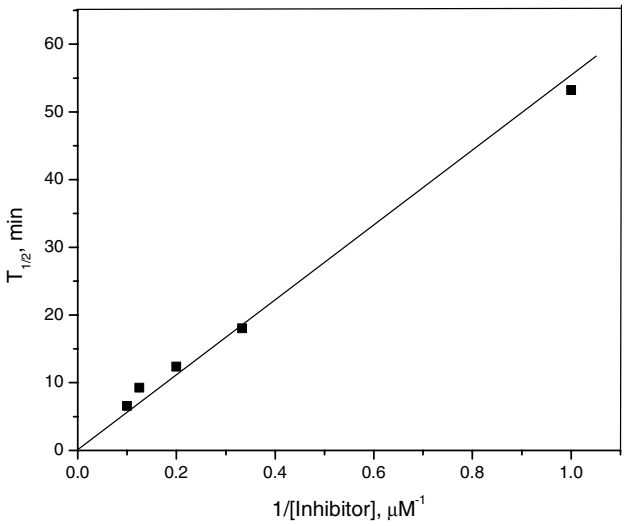


Figure 2. Kitz and Wilson replot of data in Figure 1 for inactivation of BPAO by 3-(2-quinolyl)-3-pyrroline (**5e**).

pounds **5a**, **5b**, **5e**, and **5f** are all more potent than the most potent analog **9** reported in our previous work.[10]

The *N*-methylated pyridinium derivatives **7b** and **7c** were much weaker inhibitors, and we were unable to obtain kinetic constants. Assuming that all the 3-pyrrolines inactivate through the same mechanism of cofactor derivatization, the low potency of **7b** and **7c** suggests that the permanent cationic charge results in unfavorable coulombic interactions associated with substrate binding or active-site access, since the strong electron-withdrawing nature of the pyridinium substituent would otherwise predict high inhibitory potency. A lack of steric accommodation of the *N*-methyl group is a less likely explanation, since annulation of a second ring onto the 2-pyridyl structure **5a** to give compounds **5e** and **5f** does not reduce the potency of inhibition. It appears that the pyrroline 3-aryl group is accommodated in a fairly open region of the active site.

2.3. Mechanism of enzyme inactivation: mass spectrometry supports cofactor derivatization

We had previously reported that several 3-aryl-3-pyrroline analogs are irreversible inactivators of BPAO, and that inactivation was accompanied by conversion of the TPQ quinone cofactor into a redox-inactive form.[10] In the current study, we found that preparations of BPAO inactivated by **5c**, **5d**, and **5f** failed to exhibit any redox cycling activity using the standard sodium glycinate nitroblue tetrazolium (NBT) assay.[14,15] Other analogs were not evaluated, but our finding that three new representative analogs all behaved the same way as the previously investigated 3-aryl-3-pyrrolines[10] suggests that cofactor modification is the norm for this family of inhibitor–inactivators. In addition to the loss of cofactor redox cycling activity, we provided spectroscopic evidence that inactivation of BPAO by the chromophoric analog **9** results in loss of the typical TPQ chromophore at 480 nm, with replacement by an absorption at 370 nm, consistent with the spectrum seen for the corresponding pyrroloresorcinol structures (e.g., **10a** and **10b**) generated using TPQ quinone models.[4] TPQ conversion to the **8**-derived pyrroloresorcinol was also supported by a comparison of resonance Raman spectra on the inactivated enzyme compared to TPQ model derivatives **10a** and **10b**.[4] However, because the resonance Raman data comparisons were imperfect and because the enzyme absorption at 370 nm could potentially arise from covalent attachment of the nitrophenyl-containing inhibitor to *any* position on the enzyme, we sought additional evidence for strict cofactor pyrrolization by the 3-aryl-3-pyrroline inhibitors. In this regard, we endeavored to obtain direct mass spectral evidence for the pertinent modified cofactor-containing active-site peptide.

The sequence of BPAO has been known for some time,[1] though the protein is glycosylated to a significant extent

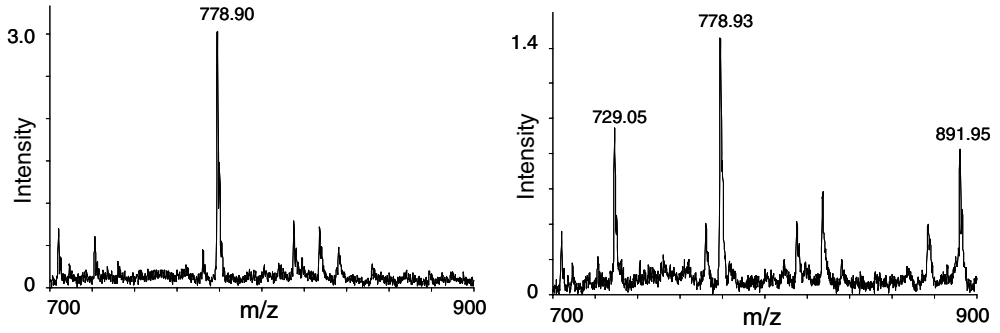


Figure 3. Negative ion MALDI-TOF spectra for the thermolytic digest of native BPAO (left panel) and BPAO inactivated by 3-(2-naphthyl)-3-pyrroline (**8**) (right panel).

on its surface. The crystal structure of the extensively deglycosylated enzyme was recently published.[16] We focused on the derivatization/inactivation of BPAO by the two highly chromophoric inhibitors 3-(2-naphthyl)-3-pyrroline (**8**) and 3-(4-methoxy-3-nitrophenyl)-3-pyrroline (**9**) (Chart 1), which would allow LC identification of peptides covalently modified in the inactivation event using both optical and mass-selective detectors. A number of attempts were made to effect efficient proteolytic digestion of the inhibitor-modified samples of purified BPAO (without deglycosylation) using trypsin, chymotrypsin, proteinase K, pepsin, and a combination of two enzymes. In part these efforts were made with the hope of establishing reasonable coverage proteolytic maps for non-active site as well as active-site regions. However, using both solution and in-gel digestion (with followup by MALDI-TOF) approaches, results were mixed, and we resorted to a thermolytic digestion strategy that was successful in first identifying the active-site TPQ cofactor following phenylhydrazine derivatization.[1] In this study, it was noted that mass spectrometric analysis of the digest without derivatization of the cofactor fails to identify it because the quinone tends to condense with released α-amino groups.[1] Through use of a modification of the published approach using thermolysin, we were able to identify the active site peptide pyrrolated by **8** by MALDI-TOF, and in the case of **9**, further identify the exact modified residue by ESI-MS/MS sequencing.

The negative ion mode MALDI-TOF spectra for the range of *m*/*z* 700–900 of the thermolytic digest of native BPAO and that following inactivation by **8** are shown in Figure 3. There are two new prominent peaks that appear in the spectrum for the modified enzyme at *m*/*z* 891.95 and 729.05. These peaks match the calculated mass for the negative ions of the cofactor-containing active site peptides Leu-Asn-X-Asp-Tyr and Leu-Asn-X-Asp, respectively, where X is the 3-(2-naphthyl)pyrrol-1-ylresorcinol derivative **11** of the TPQ cofactor. There is an additional weaker new peak at *m*/*z* 876.89, for which we have no concrete explanation at this time.

In our previous study focusing on spectroscopic characterization of BPAO inactivated by **9**,[10] we focused in the visible region where we could take advantage of resonance Raman. In the current study, for LC–MS detection of modified peptides, we focused in the UV region, where difference peaks near 270 and 310 nm were observed (along with apparent non-specific absorbance at 235 nm and below, Supplementary data). The HPLC elution was monitored at 310 to most readily select for the chromophore of interest (the lower wavelength band was deemed to be too near the strong protein absorption), and showed a major peak eluting at 71 min (Supplementary data). The corresponding mass spectrum (Supplementary data) showed a singly charged ion at *m*/*z* 919, which is consistent with the mass of the thermolytic active site peptide Leu-Asn-X-Asp-Tyr, where X corresponds to the **9**-TPQ derivative **10c**. The tandem mass spectrum of the singly charged ion at *m*/*z* 919 (Fig. 4) shows b and y ions and other internal fragments that localize to the L-N-X-D-Y sequence.

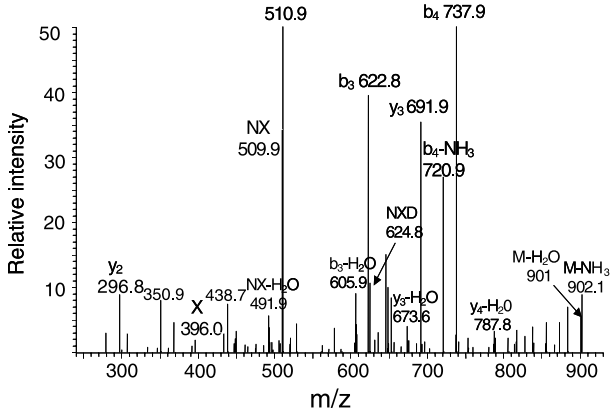
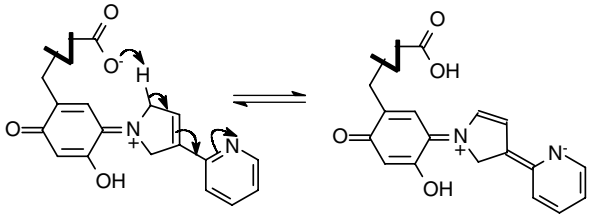


Figure 4. Collision-induced dissociation spectrum of the singly charged ion with *m*/*z* 919 for the modified active site thermolytic peptide eluting at 71 min (Supplementary data) of BPAO inactivated by 3-(4-methoxy-3-nitrophenyl)-3-pyrroline (**9**).



Scheme 4. Proposed rate-limiting step in enzymatic metabolism of 3-(2-pyridyl)-3-pyrroline (**5a**).

3. Conclusions

We have shown in this study that 3-aryl-3-pyrrolines containing an exceptionally electron-withdrawing 3-aryl group are some of the most potent mechanism-based inactivators of BPAO reported to date. Since the IC_{50} values for some of the analogs approach the enzyme concentration used in the incubation, it is possible that the true IC_{50} values would be even lower with the use of lower [E]. The high potency of these analogs likely reflects the enhanced C–H acidity, through resonance, at the key rate-limiting enzyme step where the active site base abstracts the proton on the α-C of the substrate Schiff base (see Scheme 4 for the example of **5a**). Previous studies on the substituent effect on metabolism of substituted benzylamines by BPAO have found a similar enhancement of substrate activity by acid-strengthening groups.[17]

It is curious that the 4-pyridyl analog **5c** is a much less efficient inhibitor than are the 2- and 3-pyridyl analogs **5a** and **5b**. Our interpretation of this finding is that the pyrroline 3-aryl group resides in a pocket in the enzyme active-site that results in favorable interactions of this group with the protein in the case of good substrates. For the 2- and 3-pyridyl analogs, rotation of the aryl ring about its single bond attachment to the pyrroline ring provides the possibility for the ring nitrogen to sample an arc of potential noncovalent interactions (e.g., H-bonding) in the active site, or alternatively, opportunities for avoidance of unfavorable noncovalent interactions. In contrast, the same rotation in the case of the 4-pyridyl analog results in no change in position of the ring nitrogen. Apparently, this results in a lack of favorable interaction or in an unfavorable interaction that cannot be avoided. Overall, it seems that further analog development in this family of inhibitors should focus on other electron-withdrawing substituents that lack a C_2 axis of symmetry. These compounds could be important leads to develop pharmaceutically useful inhibitors of certain copper amine oxidases, since the parent 3-phenyl compound was shown to be a substrate without inhibitory properties for the flavin-dependent mitochondrial monoamine oxidase.[10]

Supported by model studies, we previously reported strong evidence that irreversible inactivation of BPAO by 3-aryl-3-pyrrolines reflects a pyrrolization of the TPQ cofactor. Confirmatory evidence has now been provided by mass spectrometric identification of the modified active-site TPQ-containing peptide in the case of two potent analogs in this series. Although there is no guarantee that all analogs inactivate by the same mechanism, we have no evidence to suggest otherwise. Unfortunately, it is not possible to make a statement about the stoichiometry of modification from the mass spectrometric results because the native peptide is not seen. However, our previous spectroscopic study on **9** provided data consistent with a stoichiometric derivatization of the TPQ cofactor in the case of totally inactivated enzyme.[10]

4. Experimental

4.1. General procedures

NMR spectra were obtained on Varian Gemini 200 MHz (^{13}C NMR at 50 MHz) or 300 MHz (^{13}C NMR at 75 MHz) instruments, with chemical shifts referenced to the residual protonated solvent peak or sodium 2,2-dimethyl-2-silapentane-5-sulfonate in the case of D_2O. Chemical shift ranges without indicated multiplicity are given for resonances reflecting unresolved chemical shift-inequivalent (usually diastereotopic) nuclei. In the ^{13}C NMR line listings, attached proton test (APT) designations are given as (+) or (−) following the chemical shifts. High-resolution mass spectra, electron impact or fast atom bombardment (FAB) were obtained at 20–40 eV on a Kratos MS-25A instrument. Bromopyridines and bromopentafluorobenzene were purchased from commercial sources. 2-Bromoquinoline, 1-bromoisoquinoline, and ethyl 3-oxopyrrolidine-1-carboxylate (**1**) were prepared according to the indicated literature procedures. Doubly distilled water was used for all enzyme experiments. Bovine plasma amine oxidase (BPAO) (100 U/g of protein) for kinetic studies was purchased from Sigma or Worthington. Purified BPAO for NBT assay and mass spectroscopic studies was from our previous study.[10] The purity of final inhibitors evaluated (all amine salts) was assured to be >95% on the basis of their displaying a single spot on TLC and the complete absence of extraneous peaks in the ^{1}H NMR spectra.

4.2. Preparation of ethyl 3-hydroxy-3-arylpyrrolidine-1-carboxylates (2a–e)

To a solution of *n*-butyllithium (2.5 M in hexanes, 1.8 mL, 4.5 mmol) in 25 mL of either anhydrous ether (for **2a**, **2b**, **2d** and **2e**) or THF (for **2c**) was added 4 mmol of either 2-bromopyridine, 3-bromopyridine, pentafluorobromobenzene, or 2-bromoquinoline[18] in anhydrous ether (25 mL) or 4-bromopyridine in THF (25 mL) under argon at −78 °C. The reaction mixture was stirred for 2 h at −78 °C to ensure the total conversion to the corresponding aryllithium. To these solutions, ethyl 3-oxopyrrolidine-1-carboxylate[19] (**1**) (0.62 g, 4.0 mmol) in ether or THF (25 mL) was introduced dropwise at −78 °C, and the resulting mixture was stirred for an additional 3 h and then warmed to room temperature for 1 h. Saturated aqueous NH_4Cl solution (50 mL) was added to quench the reaction. The organic layer was collected and the aqueous solution was extracted with ethyl acetate (4× 50 mL). The combined organic solution was dried over anhydrous Na_2SO_4. After evaporation, the residue was purified by silica chromatography with hexanes–EtOAc as eluent to give **2a**–**f**.

Compound **2a**: eluted with EtOAc/hexanes 1:2 (77.6% yield): ^{1}H NMR (CD_3OD) δ 1.20–1.40 (br, 3H), 2.10 (m, 1H), 2.52 (m, 1H), 3.55–3.90 (4H), 4.05–4.25 (2H), 7.30 (m, 1H), 7.60–7.90 (2H), 8.53 (d, 1H, J = 4.4 Hz); ^{13}C NMR (APT, CD_3OD), there are two amide rotamers: δ 15.19 (−), 39.52 and 40.27 (+), 46.28 and

46.48 (+), 59.77 and 60.09 (+), 62.50 (+), 81.84 and 82.57 (+), 121.59 (−), 123.82 (−), 138.49 (−), 149.69 (−), 157.24 (+), 163.14 and 163.23 (+).

Compound **2b**: eluted with EtOAc/MeOH 20:1 (54.7% yield): ^{1}H NMR (CD_3OD) δ 1.27 and 1.29 (2t, 3H total, J = 7.1 Hz for both), 2.15–2.50 (2H), 3.58–3.80 (4H), 4.14 and 4.16 (2q, 2H total, J = 7.1 Hz for both), 7.45 (dd, 1H, J = 4.9, 8.0 Hz), 8.00 (dm, 1H, J = 8.0 Hz), 8.47 (br, 1H), 8.75 (br, 1H); ^{13}C NMR (APT, CD_3OD), there are two amide rotamers: δ 15.21 (−), 39.65 and 40.44 (+), 46.07 and 46.27 (+), 60.06 and 60.27 (+), 62.59 (+), 78.94 and 79.68 (+), 125.07 (−), 135.68 (−), 141.05(+), 147.68 (−), 149.08(−), 157.16 and 157.19 (+).

Compound **2c**: eluted with EtOAc/hexanes 3:1 (74.7% yield): ^{1}H NMR (CD_3OD) δ 1.27 and 1.30 (2t, 3H total, J = 7.0 Hz for both), 2.09–2.47 (2H), 3.57–3.80 (4H), 4.14 and 4.16 (2q, 2H total, J = 7.0 Hz for both), 7.60 (d, 2H, J = 6.2 Hz), 8.54 (d, 2H, J = 6.2 Hz); ^{13}C NMR (APT, CD_3OD), there are two amide rotamers: δ 15.16 (−), 39.91 and 40.71 (+), 46.16 and 46.38 (+), 60.17 and 60.40 (+), 62.59 (+), 79.61 and 80.34 (+), 122.38 (−), 150.22 (−), 141.05 (+), 155.17 and 155.28 (−), 157.11 and 157.16 (+).

Compound **2d**: eluted with a gradient of EtOAc/hexanes (1:2 to 2:1) (77% yield): mp 87–88 °C, there are two amide rotamers:^{1}H NMR ($CDCl_3$) δ 1.27 (t, 3H, J = 6.9 Hz), 2.30 (m, 1H), 2.70 (m, 1H), 3.18 and 3.45 (2s, 1H total, OH), 3.60–3.68 (3H), 4.08–4.24 (3H); ^{13}C δ 28.5, 37.7 (m) and 38.1 (m), 43.4 and 43.7, 57.6–58.1 (m), 62.0 and 62.1, 79.8, aromatic carbon signals are complicated.

Compound **2e**: eluted with a gradient of EtOAc/hexanes (1:2 to 3:1) (36.0% yield): oil, there are two amide rotamers: ^{1}H NMR ($CDCl_3$) δ 1.19–1.30 (3H), 2.14 (m, 1H), 2.40 (m, 1H), 3.66–3.88 (4H), 4.08–4.19 (2H), 5.98 and 6.00 (2s, 1H total, OH), 7.46–7.69 (2H), 7.71 (t, 1H, J = 7.0 Hz), 7.81 (d, 1H, J = 8.0 Hz), 8.05 (d, 1H, J = 8.3 Hz), 8.19 (d, 1H, J = 8.5 Hz); ^{13}C NMR ($CDCl_3$) δ 14.9 and 14.8, 46.7 and 39.8, 45.7 and 45.5, 59.7 and 59.4, 61.1, 80.6 and 79.8, 117.1, 126.9, 127.3, 127.6, 128.9, 130.2, 137.8, 146.0, 155.2, 160.19 and 160.16.

4.3. Preparation of *N*-acetyl-3-hydroxy-3-pyridylpyrrolidines (3a–c)

Each compound **2a**–**c** (0.6 g, 2.55 mmol) was dissolved in methanol (10 mL) and aqueous potassium hydroxide (5.0 M, 10 mL), and the mixture was heated at reflux for 4 h. TLC indicated complete deprotection of **2**. The methanol was removed by rotary evaporation, and acetonitrile (10 mL) was added. While keeping the reaction solution pH between 9 and 10 by periodic addition of 2 N NaOH, acetic anhydride (1.5 mL) was added over 30 min, and the reaction mixture was stirred overnight and then extracted with EtOAc/MeOH 10:1 (3× 50 mL). After removal of the organic solvent, the residue was purified by column chromatography (eluent EtOAc/MeOH 3:1) to give **3a**–**c**. NMR characterization of which indicated the presence of two amide rotamers.

Compound **3a** (88.7% yield): ^{1}H NMR (CD_3OD) δ 2.07 and 2.12 (2s, 3H total), 2.13 and 2.60 (2m, 2H total), 3.60–4.20 (4H), 7.31 (m, 1H), 7.75–7.90 (2H), 8.55 (m, 1H); ^{13}C NMR (APT, CD_3OD) δ 21.32 and 21.40 (−), 39.08 and 40.41 (+), 46.13 and 47.91 (+), 59.69 and 61.19 (+), 81.48 and 82.77 (+), 121.64 and 121.67 (−), 123.91 and 123.99 (−), 138.57 and 138.62 (−), 149.74 (−), 163.07 (+), 172.37 and 176.03 (+).

Compound **3b** (90.0% yield): ^{1}H NMR (CD_3OD) δ 1.98 and 2.12 (2s, 3H total), 2.12–2.60 (2H), 3.50–4.00 (4H), 7.53 (m, 1H), 8.15 (m, 1H), 8.48 (m, 1H), 8.76 (m, 1H). ^{13}C NMR (APT, CD_3OD) δ 21.86 and 22.35 (−), 39.16 and 40.53 (+), 45.93 and 47.65 (+), 59.76 and 61.53 (+), 78.58 and 79.90 (+), 125.09 (−), 135.70 (−), 140.70 and 140.93 (+), 147.66 and 147.71 (−), 149.15 and 149.20 (−), 172.20 and 172.43 (+).

Compound **3c** (60.8% yield): ^{1}H NMR (CD_3OD) δ 2.07 and 2.12 (2s, 3H total), 2.13 and 2.50 (2m, 2H total), 3.60–4.00 (4H), 7.71 (t, 2H, J = 6.6 Hz), 8.64 (dd, 2H, J = 1.5, 1.8 Hz).

4.4. Preparation of *N*-acetyl-3-pyridyl-3-pyrrolines (4a–c)

Each compounds **3a**–**c** (0.9 g, 3.8 mmol) was dissolved in thionyl chloride (5 mL) and stirred at room temperature for 48 h. After the removal of $SOCl_2$ in vacuo, aqueous Na_2CO_3 (5%, 10 mL) was added, and the mixture was extracted with EtOAc/MeOH 20:1 (4× 30 mL). The combined organic solvent was evaporated under reduced pressure, and the residue was purified by silica gel column chromatography to give **4a**–**c**, NMR characterization of which indicated the presence of two amide rotamers.

Compound **4a** (30.0% yield): ^{1}H NMR (CD_3OD) δ 2.12 and 2.16 (2s, 3H total), 4.40 (m, 1H), 4.60 (m, 2H), 4.80 (m, 1H), 6.66 (m, 1H), 7.31 (m, 1H), 7.65 (m, 1H), 7.82 (m, 1H), 8.60 (m, 1H); ^{13}C NMR (APT, CD_3OD) δ 21.70 and 21.97 (−), 53.85 and 54.94 (+), 55.26 and 56.32 (+), 121.95 and 122.05 (−), 124.31 (−), 125.02 and 125.27 (−), 138.31 and 138.34 (−), 139.43 and 139.46 (+), 150.25 and 150.36 (−), 152.97 (+), 171.88 (+); HRMS (EI) calcd for $C_{11}H_{13}N_2O$ (MH^+) m/z 189.1029. Found: 189.1027.

Compound **4b** (32.3% yield): ^{1}H NMR ($CDCl_3$) δ 2.05 and 2.12 (2s, 3H total), 4.38 (m, 2H), 4.55 (m, 2H), 6.15–6.30 (1H), 7.27 (m, 1H), 7.68 (m, 1H), 8.50 (m, 1H), 8.60 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 21.84 and 22.64 (−), 52.74 and 53.69 (+), 53.98 and 54.96 (+), 120.55 (−), 122.35 and 123.66 (−), 128.73 (+), 132.55 and 132.68 (−), 134.18 and 135.33 (+), 146.61 and 146.88 (−), 149.30 and 149.33 (−), 169.29 and 173.26 (+).

Compound **4c** (27.0% yield): ^{1}H NMR ($CDCl_3$) δ 2.12 and 2.16 (2s, 3H total), 4.40–4.50 (2H), 4.55–4.70 (2H), 6.41 and 6.50 (2 app p, 1H total, J = 2.1 Hz for both), 7.15–7.40 (2H), 8.40–8.70 (2H); ^{13}C NMR (CD_3OD) δ 21.84 and 22.16, 52.58 and 53.81, 55.00 and 54.94, 119.95 and 119.98, 125.08 and 125.21, 135.23 and

136.53, 139.87 and 139.93, 150.44 and 150.66, 169.09 and 169.22; HRMS (EI) Calcd for $C_{11}H_{13}N_2O$ (MH^+) *m*/*z* 189.1029. Found: 189.1027.

4.5. Preparation of 3-pyridyl-3-pyrrolines (5a–c)

Each compounds **4a**–**c** (0.06 g, 0.32 mmol) was dissolved in a mixture of ethanol (5 mL) and aqueous HCl (12 N, 5 mL), which was heated at reflux for 3 h. Evaporation to dryness afforded the pure dihydrochloride salts of **5a**–**c**.

4.5.1. 3-(2-Pyridyl)-3-pyrroline dihydrochloride (5a·2HCl). (98.0% yield): mp 223–225 °C; ^{1}H NMR (D_2O) δ 4.47 (dd, 2H, J = 2.6, 3.6 Hz), 4.67 (dd, 2H, J = 2.5, 3.7 Hz), 7.05 (app p, 1H, J = 2.2 Hz), 7.96 (dd, 1H, J = 1.1, 6.0 Hz), 8.03(d, 1H, J = 8.1 Hz), 8.57 (ddd, 1H, J = 1.6, 2.2, 8.0 Hz), 8.70 (dd, 1H, J = 1.6, 6.0 Hz); ^{13}C NMR (APT, D_2O) δ 65.87 (+), 68.45 (+), 140.24 (−), 141.14 (−), 144.14 (+), 146.85 (−), 156.73 (−), 158.27 (+), 162.08 (−); HRMS (FAB) Calcd for $C_9H_{11}N_2$ (MH^+) *m*/*z* 147.0922. Found: 147.0927.

4.5.2. 3-(3-Pyridyl)-3-pyrroline dihydrochloride (5b·2HCl). (68.7% yield): mp 252–254 °C; ^{1}H NMR (D_2O) δ 4.37 (dd, 2H, J = 2.4, 3.7 Hz), 4.57 (dd, 2H, J = 2.4, 3.6 Hz), 6.73 (app p, 1H, J = 2.2 Hz), 8.08 (dd, 1H, J = 5.8, 7.0 Hz), 8.73 (m, 2H), 8.88(d, 1H, J = 1.2 Hz); ^{13}C NMR (APT, D_2O) δ 66.09 (+), 68.11 (+), 141.18 (−), 142.29 (−), 145.56 (+), 146.33 (+), 153.42 (−), 155.21 (−), 158.48 (−); HRMS (FAB) Calcd for $C_9H_{11}N_2$ (MH^+) *m*/*z* 147.0922. Found: 147.0923.

4.5.3. 3-(4-Pyridyl)-3-pyrroline dihydrochloride (5c·2HCl). (98.7% yield): mp 227–228 °C; ^{1}H NMR (free base in CD_3OD) δ 4.03 (s, 2H), 4.17 (br, 2H), 6.7 (m, 1H), 7.47 (d, 2H, J = 3.1 Hz), 8.73 (d, 2H, J = 3.1 Hz); ^{13}C NMR (APT, CD_3OD) δ 53.51 (+), 55.18 (+), 122.04 (−), 129.21 (−), 138.79 (+), 142.97 (+), 150.46 (−), 150.53 (−); HRMS (FAB) calcd for $C_9H_{11}N_2$ (MH^+) *m*/*z* 147.0922. Found: 147.0927.

4.6. Preparation of *N*-acetyl-3-(*N*-methylpyridyl)-3-pyrrolines (6b,c)

Methyl iodide (2 mL) was added to a solution of **4b** or **4c** (0.075 g, 0.40 mmol) in CH_2Cl_2 (5 mL), and the reaction mixture was stirred for 36 h at room temperature. The slight yellow solid was collected and washed thoroughly with CH_2Cl_2 to give methiodides **6bc** in stoichiometric yield, NMR characterization of which indicated the presence of two amide rotamers.

Compound **6b**: ^{1}H NMR (CD_3OD) δ 2.12 and 2.21 (2s, 3H total), 4.46 (s, 3H), 4.48 (s, 2H), 4.65 (s, 2H), 6.90 (m, 1H), 8.09 (m, 1H), 8.69 (d, 1H, J = 8.6 Hz), 8.84 (d, 1H, J = 3.6 Hz), 9.16(d, 1H, J = 8.0 Hz).

Compound **6c**: ^{1}H NMR (DMSO) δ 2.02 and 2.06 (2s, 3H total), 4.29 (s, 3H), 4.37 (m, 1H), 4.54 (m, 1H), 4.59 (m, 1H), 4.75 (m, 1H), 7.30 (m, 1H), 8.16–8.20 (2H), 8.92–8.98 (2H).

4.7. Preparation 3-(*N*-methylpyridyl)-3-pyrrolines (7b–c)

Compounds **6b**–**c** were heated in a 1:1 mixture of 12 N HCl and ethanol at reflux for 3 h, followed by complete removal of solvent to give **7b**–**c** iodide hydrochloride salts.

Compound **7b$^+$I**·HCl (yield 71.9%): mp 196–197 °C; ^{1}H NMR (D_2O) δ 4.41 (s, 3H), 4.43 (d, 2H, J = 2.2 Hz), 4.60 (d, 2H, J = 2.2 Hz), 6.78 (t, 1H, J = 2.2 Hz), 8.06 (dd, 1H, J = 6.2, 8.1 Hz), 8.58 (d, 1H, J = 8.1 Hz), 8.74 (d, 1H, J = 6.1 Hz), 8.97 (s, 1H); ^{13}C NMR (APT, D_2O) δ 63.45 (−), 66.32 (+), 68.28 (+), 141.64 (−), 142.82 (−), 145.43 (+), 146.88 (+), 156.78 (−), 157.60 (−), 159.19 (−); HRMS (FAB) calcd for $C_{10}H_{13}N_2$ (M^+) *m*/*z* 161.1080. Found: 161.1079.

Compound **7c$^+$I**·HCl (yield 93.9%): mp 193–194 °C; ^{1}H NMR (CD_3OD) δ 4.39 (s, 3H), 4.46 (d, 2H, J = 7.2 Hz), 4.62 (s, 2H), 7.21 (s, 1H), 8.18 (d, 1H, J = 6.0 Hz), 8.90 (d, 1H, J = 6.0 Hz); ^{13}C NMR (CD_3OD) δ 148.2, 147.0, 134.5, 133.9, 125.6, 54.9, 52.5, 48.0; HRMS (EI) Calcd for $C_{10}H_{13}N_2$ (M^+) *m*/*z* 161.1080. Found: 161.1079.

4.8. Preparation of 3-pentafluorophenyl-3-pyrroline hydrochloride (5d·HCl)

Compound **2d** was subjected to $SOCl_2$-mediated dehydration according to the above procedure for conversion of compounds **3**–**4**. Without purification, *N*-(ethoxycarbonyl)-3-pyrroline **4d** (0.5 mmol) was dissolved in HBr (48%, 10 mL) and acetic acid (3 mL), and the mixture was heated at reflux for 3 h. After removal of all the solvent, the residual solid was coevaporated with HCl (37.5%, 20 mL) to dryness three times by rotary evaporation. The resulting residue was recrystallized from EtOH to Et_2O to yield **5d** hydrochloride (85.0% yield): mp 203–204 °C; ^{1}H NMR (CD_3OD) δ 4.35 (s, 2H), 4.52 (s, 2H), 6.56 (s, 1H); ^{13}C (CD_3OD) δ 53.5, 53.8 (t, J = 4.2 Hz), other carbon signals are complicated by ^{19}F splitting; HRMS (FAB) calcd for $C_{10}H_6F_5N$ (MH^+) *m*/*z* 236.0499. Found: 236.0497.

4.9. Preparation of 3-(2-quinolyl)-3-pyrroline dihydrochloride (5e·2HCl)

Compound **2e** was subjected to $SOCl_2$-mediated dehydration according to the above procedure for conversion of compound **3**–**4**. After removal of excess $SOCl_2$, neutralization (Na_2CO_3), extraction (EtOAc), and silica gel chromatographic purification (eluent EtOAc/hexanes 2:1) yielded *N*-(ethoxycarbonyl)-3-pyrroline **4e** as an oil in 40.0% yield, NMR characterization of which indicated the presence of two amide rotamers: ^{1}H NMR (CD_3OD) δ 1.26–1.34 (m, 3H), 4.12–4.20 (m, 2H), 4.30 (s, 2H), 4.50–4.57 (m, 2H), 6.61 and 6.64 (2s, 1H total), 7.52 (t, 1H, J = 7.4 Hz), 7.68–7.72 (2H), 7.81 (d, 1H, J = 7.8 Hz), 7.96 (dd, 1H, J = 5.2, 8.3 Hz), 8.15 (d, 1 H, J = 8.7 Hz); ^{13}C NMR (CD_3OD) δ 15.1, 54.1 and 54.4, 55.2 and 55.5, 62.7 and 62.8, many aryl peaks. Compound **4e** (0.5 mmol) was dissolved in HBr (48%, 10 mL) and acetic acid (3 mL), and the mixture was heated at reflux for 3 h. After removal of all the

solvent, the residual solid was coevaporated with HCl (37.5%, 20 mL) to dryness three times by rotary evaporation. The resulting residue was recrystallized from EtOH to Et_2O to yield **5e** dihydrochloride (82.0%): mp 280 °C (dec); ^{1}H NMR (CD_3OD) δ 4.55 (d, 2H, J = 2.3 Hz), 4.85 (d, 2H, J = 2.0 Hz), 7.47 (m, 1H), 7.93 (t, 1H, J = 7.5 Hz), 8.12 (dt, 1H, J = 8.4, 1.3 Hz), 8.22 (d, 1H, J = 8.7 Hz), 8.28 (d, 1H, J = 8.4 Hz), 8.46 (d, 1H, J = 8.4 Hz), 9.06 (d, 1H, J = 8.7 Hz); ^{13}C NMR (CD_3OD) δ 52.6, 54.9, 121.7, 122.9, 129.7, 130.2, 131.1, 132.9, 136.1, 136.4, 140.8, 147.1, 148.4; HRMS (FAB) Calcd for $C_{13}H_{12}N_2$ (MH^+), 197.1079. Found: 197.1082.

4.10. Preparation of 3-(1-isoquinolyl)-3-pyrroline dihydrochloride (5f·2HCl)

The lithium reagent from 1-bromoisoquinoline[20] was prepared in diethyl ether at −78 °C and reacted with ethyl 3-oxopyrrolidine-1-carboxylate as described for **2a**–**e** above. After workup, the resulting *N*-(ethoxycarbonyl)-3-hydroxypyrrolidine **2f** was subjected without purification to $SOCl_2$-mediated dehydration. After removal of excess $SOCl_2$, neutralization (Na_2CO_3), extraction (EtOAc), and silica gel chromatographic purification (eluent EtOAc/hexanes 2:1) yielded *N*-(ethoxycarbonyl)-3-pyrroline **4f** as an oil in 19% overall yield, NMR characterization of which indicated the presence of two amide rotamers: ^{1}H NMR ($CDCl_3$)δ 1.27–1.36 (m, 3H), 4.18–4.26 (m, 2H), 4.48–4.59 (m, 2H), 4.79–4.85 (m, 2H), 6.20–6.40 (m, 1H), 7.57–7.86 (m, 4H), 8.31–8.60 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 14.9 and 15.0, 54.4 and 54.9, 55.2 and 55.8, 61.20 and 61.24, many aryl peaks. Compound **4f** (0.5 mmol) was dissolved in HBr (48%, 10 mL) and acetic acid (3 mL), and refluxed for 3 h. After removal of all the solvent, the residue solid was coevaporated with HCl (37.5%, 3× 20 mL) to dryness by rotary evaporator. The resulting residue was recrystallized from EtOH to Et_2O to yield **5f** dihydrochloride (87.0%): mp 208 °C (dec); ^{1}H NMR (CD_3OD) δ 4.61 (s, 2H), 4.83 (s, 2H), 6.92 (s, 1H), 8.12 (t, 1H, J = 7.4 Hz), 8.28 (t, 1H, J = 8.1 Hz), 8.39 (d, 1H, J = 8.1 Hz), 8.52–8.68 (m, 3H); ^{13}C NMR (CD_3OD) δ 54.9, 55.0, 127.1, 127.9, 129.6, 129.8, 132.4, 133.0, 137.5, 138.0, 141.0, 151.0, 158.6; HRMS (FAB) Calcd for $C_{13}H_{12}N_2$ (MH^+), 197.1097. Found: 197.1080.

4.11. Enzymology

Time-dependent inactivations of BPAO by candidate inhibitors, determination of active enzyme concentration in the various preparations, determinations of irreversibility, and the NBT assays for cofactor redox cycling activity were conducted as previously described,[15] except that the pH 7.2 sodium phosphate buffer concentration was 50 mM rather than 100 mM.

4.12. Enzyme modification for mass spectrometric studies

Purified BPAO[10] (2 mg/mL) in 0.1 M potassium phosphate buffer, pH 7.4, was incubated with **8** or **9** (1 mM) in a total volume of 0.5 mL at 30 °C for 2 h. The samples were then denatured by the addition of solid urea and a 0.5 M aqueous solution of dithiothreitol (DTT) to reach final concentrations of 6 M and 10 mM, respectively, incubating at 37 °C for 1 h. Iodoacetamide (0.5 M aqueous stock) was then added to a final concentration of 40 mM, with incubation at room temperature in the dark for 1 h. Excess DTT solution was then added (to reach 50 mM) to consume unreacted iodoacetamide. The sample was then dialyzed against 10 mM NH_4HCO_3 in a cold room with three changes in the buffer over 24 h. After dialysis, the protein was digested by thermolysin at a ratio of BPAO:thermolysin of 50:1 (w/w) for 24 h at 37 °C. The same amount of thermolysin was added a second time (bringing the final ratio to 25:1), with an additional 24-h incubation at 37 °C. The final sample was concentrated to a volume of 50 μL.

4.13. HPLC–ESI-MS/MS analysis for modification by 9

Aliquots (20 μL) of peptides were separated using a Michrom Magic 2002 HPLC (Michrom BioResources, Auburn, CA) equipped with a Varian Vydac C18 1× 250 mm column. The eluate was monitored at 235 and 310 nm, with a binary gradient elution system of solvent mixtures A (2% aqueous acetonitrile containing 0.05% TFA) and B (90% aqueous acetonitrile containing 0.05% TFA) according to the following 2-h program: 100% A for 8 min, 98% A for 7 min, 95% A for 45 min, 84% A for 20 min, 65% A for 10 min, 0% A (100% B) for 18 min, 100% A for 12 min, with a flow rate of 100 μL/min for the first 95 min and 150 μL/min for the last 25 min. The eluate from the column was transferred to a Finnigan LCQDuo Mass Spectrometer (Thermo Electron, San Jose, CA); the ESI was programmed with the following settings: nitrogen sheath gas flow rate (arbitrary units) at 50; auxiliary gas flow rate at 0; capillary temperature at 160 °C; spray voltage at 4.4 kV. The mass spectrometer was programmed for selected reaction monitoring and MS/MS, and the normalized collision energy was set to 40%, the tube lens offset at 30 V. Other parameters were adjusted using a tune method developed using model compound **10a**.

4.14. MALDI-TOF analysis for modification by 8

The protein digest samples were desalted and eluted using C18 Millipore Ziptips (Millipore, Bedford, MA) according to the manufacturer's recommendations. Briefly, the Ziptip was wet by 50% acetonitrile in distilled–deionized water and thereafter equilibrated by 0.1% trifluoroacetic acid (TFA). Then the peptides were extracted with the Ziptip and washed with 0.1% TFA. Finally the sample was eluted with 2 μL of a solution of α-cyano-4-hydroxycinnamic acid (20 mg/mL in 70% acetonitrile and 0.1% TFA) directly onto the stainless steel target and allowed to dry at room temperature. The mass spectrometer used was a Bruker BiFlex III MALDI-TOF instrument (Bruker Daltonics, Bremen, Germany) equipped with a pulsed nitrogen laser (3 ns pulse at 337 nm). Spectra were collected in the negative ion and reflectron mode with an average of 250 laser shots.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2006.11.025.

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