Bioorganic & Medicinal Chemistry 22 (2014) 1558–1567

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthesis of 2,6-disubstituted benzylamine derivatives as reversible selective inhibitors of copper amine oxidases

Francesco Lucchesini [a], Marco Pocci [a], Silvana Alfei [a], Vincenzo Bertini [a,*], Franca Buffoni [b,†]

[a] Dipartimento di Farmacia, Università di Genova, Via Brigata Salerno, 13, 16147 Genova, Italy
[b] Dipartimento di Farmacologia Preclinica e Clinica, Università di Firenze, Viale G. Pieraccini, 6, 50139 Firenze, Italy

ARTICLE INFO

Article history:
Received 14 September 2013
Revised 29 December 2013
Accepted 21 January 2014
Available online 30 January 2014

Keywords:
Copper amine oxidases
Reversible inhibitors
2,6-Disubstituted benzylamines
Metalation
Formylation

ABSTRACT

In order to obtain substrate-like inhibitors of copper amine oxidases (CAOs), a class of enzymes involved in important cellular processes as well as in crosslinking of elastin and collagen and removal of biogenic primary amines, we synthesized a set of benzylamine derivatives properly substituted at positions 2 and 6 and studied their biological activity towards some members of CAOs.

With benzylamines **6**, **7**, **8** containing linear alkoxy groups we obtained reversible inhibitors of benzylamine oxidase (BAO), very active and selective toward diamine oxidase (DAO), lysyl oxidase (LO) and monoamine oxidase B (MAO B) characterized by a certain toxicity consequent to the crossing of the brain barrier. Poorly toxic, up to very active, reversible inhibitors of BAO, very selective toward DAO, LO and MAO B, were obtained with benzylamines **10**, **11**, **12** containing hydrophilic ω-hydroxyalkoxy groups. With benzylamines **13**, **14**, **15**, containing linear alkyl groups endowed with steric, but not conjugative effects for the absence of properly positioned oxygen atoms, we synthesized moderately active inhibitors of BAO reversible and selective toward DAO, LO and MAO B.

The cross examination of the entire biological data brought us to the conclusion that the bioactive synthesized compounds most likely exert their physiological role of reversible inhibitors in consequence of the formation of a plurality of hydrogen bonds or hydrophobic non-covalent interactions with proper sites in the protein. Accordingly, the reported inhibitors may be considered as a set of research tools for general biological studies and the formation of enzyme complexes useful for X-ray structure determinations aimed at the design of more sophisticated inhibitors to always better modulate the protein activity without important side effects.

1. Introduction

Copper amine oxidases[1] (CAOs, EC 1.4.3.6) are ubiquitous enzymes which control important cellular processes as well as crosslinking of elastin and collagen, by catalyzing the oxidative deamination of primary amines RCH_2NH_2 according to Eq. (1).

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCH{=}O + NH_3 + H_2O_2 \quad (1)$$

Pluridecennial studies and several X-ray structures of copper amine oxidases from bacteria [*Escherichia coli* amine oxidase (ECAO)[2] and *Arthrobacter globiformis* amine oxidase (AGAO)[3]], yeast [*Hansenula polymorpha* amine oxidase (HPAO)[4] and *Pichia pastoris* lysyl oxidase (PPLO)[5]], plants [*Pisum sativum* pea seedling amine oxidase (PSAO)][6] and mammals [*Homo sapiens* diamine oxidase (hDAO)][7] allowed to ascertain that all CAOs are homodimers having one copper ion Cu^{2+} and one quinone cofactor per subunit, and variable percentages of a carbohydrate portion depending on the enzyme source.

For the various typologies of CAOs, excluding lysyl oxidase (LO), the cofactor is 2,4,5-trihydroxyphenylalanine quinone (TPQ) which is connected to the protein through one covalent bond enabling TPQ to assume two conformations, active and inactive, influencing the enzyme reaction mechanism. For LO the cofactor is lysine tyrosylquinone (LTQ)[8] stably connected to the protein through two covalent bonds.

Different typologies of CAOs have 'substrate channels' of different dimensions, from very narrow to very wide, in agreement with the needs of their preferential substrates.

The X-ray structures of CAOs and of several complexes of CAOs with inhibitors of different types, together with the bulk of results from previous studies, allowed to conceive a reasonable pathway for a ping-pong enzyme reaction mechanism corresponding to a sequence of reductive and oxidative half reactions. In the reductive half reaction the substrate amine reacts with TPQ to form a

* Corresponding author. Tel.: +39 010 3532685.
E-mail address: bertini@difar.unige.it (V. Bertini).
† Professor Emeritus of the University of Florence, deceased on November 1, 2012.

http://dx.doi.org/10.1016/j.bmc.2014.01.037
0968-0896/

quinoneimine (substrate Schiff base) in tautomeric equilibrium with a quinoaldimine (product Schiff base) which hydrolyzes to release aldehyde and to produce the reduced aminoresorcinol form of TPQ (Scheme 1).

In the oxidative half reaction the reduced cofactor in the presence of molecular oxygen is reoxidized to TPQ with releasing of hydrogen peroxide and ammonia (Scheme 2).

Inhibitors of different structure succeed in trapping CAOs in stable complexes through different mechanisms of action mainly disclosed through X-ray structure determinations as cited in the following examples.

The 2-hydrazinopyridine inhibits ECAO by covalently binding at position 5 of the quinone ring of TPQ in a manner similar to a substrate, but the produced hydrazone analogue to a substrate Schiff base, having a nitrogen atom in place of a CH group, prevents any proton abstraction, thus trapping the enzyme in a covalent complex.[9]

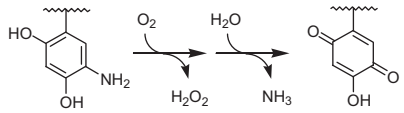
With AGAO the inhibitor 4-(2-naphthyloxy)-2-butyn-1-amine begins to react as a substrate producing aldehyde and transforming the cofactor into the 5-amino form of the reduced TPQ, but such amino group binds covalently to the triple bond present in the aldehydic product giving a stable system which does not allow enzyme regeneration.[10]

Among the two *trans* enantiomers *(1S,2R)*-(+)-*trans*-2-phenylcyclopropylamine [(+)-TCP] and *(1R,2S)*-(−)-*trans*-2-phenylcyclopropylamine [(−)-TCP] only the first one inhibits ECAO. Such enantiomer can place itself into the substrate channel and form the Schiff base at position 5 of TPQ, but the hydrogen on the carbon of the cyclopropyl ring adjacent to the nitrogen linked to TPQ, pointing away from the proton abstraction site on the protein chain, cannot be removed and the enzyme reaction is blocked.[11] Nevertheless, extensive dialysis of the inhibited enzyme removed TCP, restoring the enzyme activity and indicating that binding is reversible.[12]

Berenil [1,3-bis(4′-amidinophenyl) triazene] and Pentamidine [1,5-bis(4-amidinophenoxy) pentane] are excellent inhibitors of hDAO through non-covalent binding.[7] They occupy the narrow asymmetrical cone-shaped cavity of the substrate channel adopting a conformation essentially straight for Berenil and horseshoe-shaped for Pentamidine, blocking the access of substrates to TPQ. Though not covalently bonded to the protein, these substances form a very stable system based on hydrogen bonds and hydrophobic interactions.

Two racemic aryl 2,3-butadienamine analogues, such as 5-phenoxy-2,3-pentadienylamine (POPDA) and 6-phenyl-2,3-hexadienylamine (PHDA), are inhibitors of AGAO.[13] Initially they react with AGAO as substrates giving the corresponding aldehyde and the 5-amino form of the reduced TPQ, but the amino group further reacts with the activated system of the allene aldehydes attacking different carbon atoms either with POPDA or PHDA. In both cases, trapping TPQ in covalent complexes causes the inactivation of the enzyme.

Three hydrazine derivatives, such as benzylhydrazine (BHZ), 4-hydroxybenzylhydrazine (4-OH-BHZ) and phenylhydrazine (PHZ), structural analogues of 2-phenylethylamine, tyramine and benzylamine proved to be the first two good substrates of AGAO and the last poor. The X-ray structures of complexes of these inhibitors with AGAO evidenced the formation of covalent hydrazone



Scheme 2. Oxidative half-reaction of TPQ cofactor.

adducts with TPQ, structural analogous to the substrate Schiff base of TPQ[14] in accordance with the behaviour of 2-hydrazinopyridine towards ECAO (see above). The comparative study of the structures of such adducts with the three different hydrazine derivatives provided relevant structural insights into the substrate specificity of AGAO.

All the above reports prove the current and prominent interest for new inhibitors of CAOs as tools for disclosing the complex biological role of each of these enzymes often endowed with broad substrate specificity adapted to a variety of physiological needs. In view of pharmaceutical use of CAOs inhibitors, especially if irreversible, such peculiar substrate specificity entails severe risk of a complete enzyme inactivation which insidiously can open the way to noxious interferences.[7,15–19]

In this light, reversible inhibitors of CAOs forming rather stable complexes with the protein on the basis of non-covalent interactions give rise to relevant expectations for bioactive molecules able to selectively modulate the protein activity, avoiding the possible risks inherent in irreversible inhibitors.[7,20]

Some of us prepared and studied[21] the first substrate-like, very active, fully reversible inhibitors of different CAOs corresponding to derivatives of 4-aminomethylpyridine with alkoxy (Series 1), alkylthio (Series 2) and alkylamino groups (Series 3) at positions 3 and 5 of the ring, or with alkylamino groups at position 3 (Series 4).

The inhibitory activity of the prepared compounds was tested on different CAOs such as diamine oxidase of porcine kidney (DAO), benzylamine oxidase of porcine serum (BAO), lysyl oxidase of porcine aorta (LO), pea seedling amine oxidase (PSAO), *Hansenula polymorpha* amine oxidase (HPAO), and FAD monoamine oxidases of rat liver (MAO A and MAO B).

Series 1 contained reversible, very active inhibitors of BAO, selective with respect to DAO, LO, PSAO, HPAO, MAO A and MAO B.

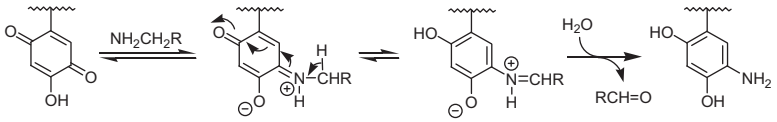
Series 2 contained reversible inhibitors of BAO very selective with respect to DAO and MAO, unexpectedly affording an interesting new type of good substrate of DAO.

Series 3 allowed to suppress the selectivity especially between BAO and DAO, containing reversible inhibitors very active on both enzymes and active on MAO A, MAO B, PSAO and HPAO.

Series 4 contained reversible non-selective inhibitors very active on DAO, BAO and PSAO, and active on HPAO, MAO A and MAO B.

Kinetic experiments on the enzymatic reactions performed with 4-aminomethylpyridine and benzylamine as substrates of DAO and BAO showed an interaction for the pyridine ring stronger than that of benzene ring with both the enzymes.

It is interesting to note that the fully reversible inhibitor 3,5-diethoxy-4-aminomethylpyridine is well absorbed orally in rats with a bioavailability of 51.5% undergoing a rather fast transformation with a blood half-life of 2.0 h.[22]



Scheme 1. Reductive half-reaction of TPQ cofactor.

It is reasonable to assume that the heterocyclic nitrogen atom of 4-aminomethylpyridine allows the pyridine ring to place itself in proximity of the enzymatic active site differently from how the benzene ring of benzylamine does, affecting both the reactivity of the two substrates and the activity of substrate-like inhibitors derived from them.

Since the study of such inhibitors and the comparison of their structures and behaviours appeared to us worthy of attention, in this work we report syntheses and properties of a logical set of 2,6-disubstituted benzylamines, consisting of very selective, very active, reversible inhibitors of BAO from porcine serum which has benzylamine as preferred substrate, like other CAOs such as plasma amine oxidase (PAO) and tissular semicarbazide sensitive amine oxidase (SSAO).

The set of the biological data characterizes the prepared inhibitors as interesting bioactive materials for biological studies and specifically for the preparation of a variety of stable crystalline adducts with proper CAOs for X-ray structure determinations.

2. Results and discussion

In search for substrate-mimicking inhibitors of those CAOs for which benzylamine is a good substrate, we modified the benzylamine structure by introducing substituents in the benzene ring at positions *ortho* to the aminomethyl group, and recorded significant inhibitory activities.[23] On that basis, we successively planned the present study concerning mono- and disubstituted benzylamines having at positions 2 and 6, either hydrocarbon substituents with expected steric effects, or alkoxy substituents capable of both steric and coordinative effects due to the presence of oxygen atoms.

Both adequate hydrocarbon chains and properly positioned oxygen atoms resulted essential for a good inhibitory action and a good control, through the balance of hydrophobic and hydrophilic groups, of the toxic effect connected to the crossing of the blood-brain barrier.

Chart 1 summarizes the structures of the benzylamine derivatives discussed in this paper.

2.1. Chemistry

2,6-Dihydroxybenzylamine hydrochloride (**4**) was obtained from the known 2,6-dimethoxybenzylamine (**5**)[24] through demethylation with hydrobromic acid at reflux followed by halide exchange with concentrated hydrochloric acid (Scheme 3).

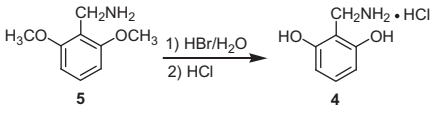
2,6-Dialkoxybenzylamine hydrochlorides **6**–**9** were synthesized through metalation of the corresponding 1,3-dialkoxybenzenes **18**–**21** in THF, followed by formylation,[25] conversion into oximes **22a**–**25a**, reduction of oximes with Raney alloy and reaction with gaseous HCl (Scheme 4).

AlH_3[26] was explored as an alternative reducing agent of the oxime function, but when employed in a test reduction of **22a** it caused partial elimination of the ethoxy group with formation of 2-ethoxybenzylamine and it was abandoned.

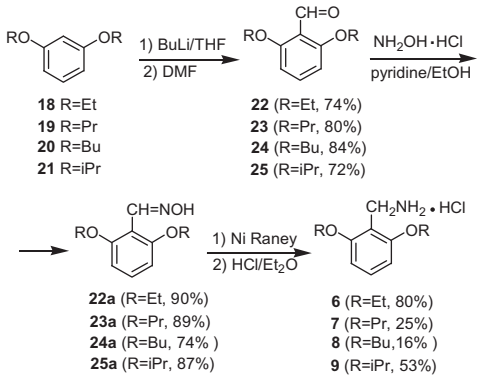
The synthesis of 2,6-bis(ω-hydroxyalkoxy)benzylamines hydrochlorides **10**–**12** was carried out according to Scheme 5, through dialkylation of resorcinol with the appropriate 2-(ω-bromoalkoxy)tetrahydropyran to obtain **26**, **27** and **28**, metalation with BuLi in diethyl ether, formylation to afford benzaldehydes **29**,[27] **30** and **31** in moderate yields, and reductive amination followed by reaction with gaseous HCl in THF. The reductive amination of benzaldehydes **29**–**31** was necessary in this case since the conversion to the corresponding oximes proved difficult.

In order to improve the metalation/formylation step, **26** was made to react with BuLi and DMF in THF,[25] but the formation of products which did not contain any formyl group was recorded, presumably originating from reactions involving the 2-(2-tetrahydropyranyloxy)ethoxy substituent (Scheme 6).

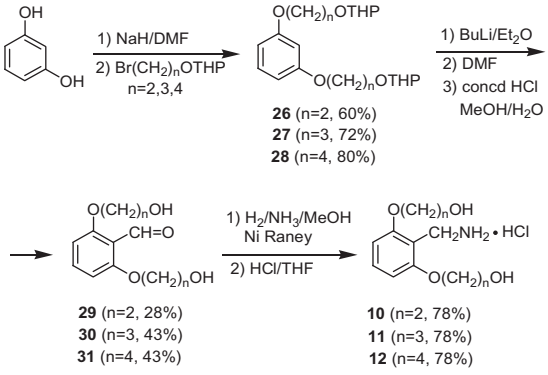
Reasonably, vinylether **32** was obtained by β-elimination of tetrahydropyranyl anion promoted by BuLi,[27] while diol **33** appeared



Scheme 3. Synthesis of inhibitor **4**.



Scheme 4. Synthesis of inhibitors **6**–**9**.

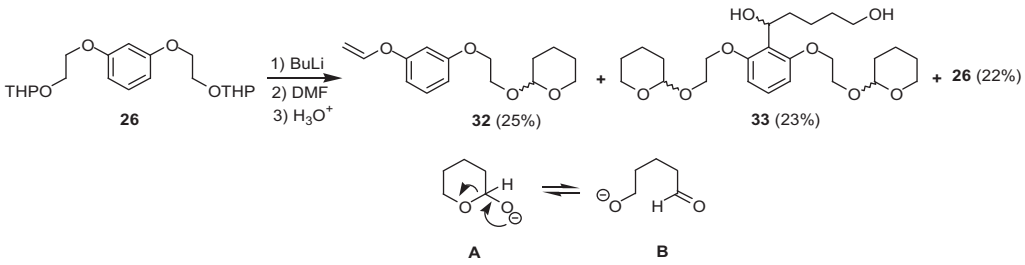


Scheme 5. Synthesis of inhibitors **10**–**12**.

1 (R=OH), **2** (R=OEt), **3** (R=CH_2OH)

4 (R=OH), **5** (R=OMe), **6** (R=OEt), **7** (R=OPr), **8** (R=OBu), **9** (R=OiPr),**10** (R=OCH_2CH_2OH), **11** (R=$OCH_2CH_2CH_2OH$), **12** (R=$OCH_2CH_2CH_2CH_2OH$), **13** (R=Et), **14** (R=Pr), **15** (R=Bu), **16** (R=CH_2OCH_3), **17** (R=$CH_2OCH_2CH_3$)

Chart 1. Molecular structures of the prepared inhibitors.



Scheme 6. Formation of side-products **32** and **33** in metalation of **26**.

to originate from reaction of metalated **26** and the carbonyl form B of the eliminated tetrahydropyranyl anion A (Scheme 6)[28] (see Supplementary data for proposed reaction schemes, experimental details and NMR assignments).

The synthesis of hydrochloride **13** was carried out without difficulty from 2,6-diethylbenzonitrile[29] (Scheme 7).

For the synthesis of hydrochlorides **14** and **15** we resorted to benzaldehydes **34** and **35** as key intermediates (Scheme 8). The new benzaldehyde **35** was prepared in analogy to known **34**[30] through a three step sequence exploiting 2-(2,6-difluoro phenyl)-4,5-dihydro-4,4-dimethyl-1,3-oxazole[31] (Supplementary data).

Scheme 9 shows the synthesis of the hydrochlorides **16**–**17** starting from 2,6-bis(bromomethyl)benzonitrile.[32]

2.2. Biology

The activity of benzylamines **1**–**17** as substrate-mimicking inhibitors of CAOs was assayed with different copper amine oxidases such as BAO from porcine serum having benzylamine as a preferred substrate, DAO from porcine kidney having putrescine, cadaverine, histamine as preferred substrates, LO from porcine aorta having lysine residues contained in elastine or collagen as preferred substrate, and also with the FAD monoamine oxidase MAO B from rat liver which counts among its substrates 2-phenylethylamine and benzylamine.

Table 1 summarizes the results of the biological assays.

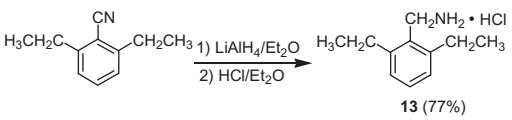
Monosubstituted benzylamines **1**–**3**, and benzylamine derivative **4** substantially did not show any inhibitory activity (Table 1, Chart 1).

The inhibitory activities of benzylamines **5**–**17** (Table 1, Chart 1) towards DAO, LO and the FAD enzyme MAO B were negligible, showing IC_{50} values always higher than 10^{-3} mol/L with the exception of value 1.9×10^{-4} mol/L corresponding to a modest activity of compound **5** towards MAO B.

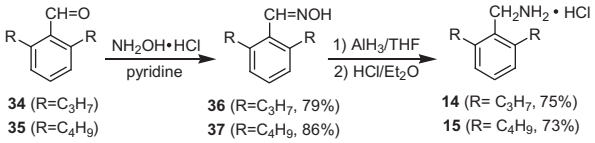
On the contrary, several 2,6-disubstituted benzylamines were active towards BAO at a concentration as low as 10^{-7} down to 6.6×10^{-8} mol/L corresponding to very active, very selective and reversible inhibitors.

The introduction of alkoxy groups at positions 2 and 6 of benzylamine produced selective inhibitors of BAO with an inhibitory activity that was modest in the case of the small methoxy groups (**5**), but in the case of longer aliphatic chains as ethoxy (**6**), propoxy (**7**) and butoxy groups (**8**) suddenly increased ($IC_{50} \sim 10^{-7}$ mol/L), the values recorded representing the maximum inhibition limit for such type of compounds.

It is remarkable that the inhibitory activity dropped (IC_{50} $>10^{-3}$ mol/L), in the presence of bulkier branched alkoxy groups



Scheme 7. Synthesis of inhibitor **13**.



Scheme 8. Synthesis of inhibitors **14** and **15**.

such as the isopropoxy of **9**, probably sterically unsuitable for the substrate channel of the enzyme active site.

For compounds **5**–**8** the toxicity increased, and the LD_{50} decreased with the growth of the hydrophobic character of the alkoxy groups, being accompanied by convulsive effects on the treated animals before death, in consequence of the brain barrier crossing.

The introduction at positions 2 and 6 of benzylamine of ω-hydroxyalkoxy groups having a good hydrophilic character (**10**–**12**, Table 1, Chart 1), gave poorly toxic, very active, reversible inhibitors of BAO with IC_{50} down to 6.6×10^{-8} mol/L. These compounds endowed with peripheral OHs, were considered also attractive for the possibility of anchoring the inhibitor to polymers or resins through one of the hydroxyl groups with a good chance of preserving its activity, in analogy with what we obtained with the synthesis of the bioactive resins of 2-methoxy-6-[(4-vinyl)benzyloxy]benzylamine hydrochloride.[33]

The 2,6-dialkylbenzylamines **13**, **14** and **15** proved reversible and selective inhibitors of BAO, but significantly less active than the dialkoxy derivatives **6**, **7** and **8** which can profit from conjugative effects of the oxygen atoms bonded to the benzene ring.

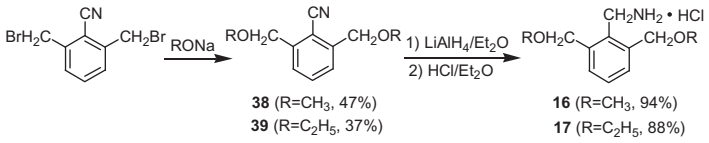
The 2,6-dialkoxymethylbenzylamines **16** and **17**, bearing substituents with the same number of carbon and oxygen atoms as **6** and **7**, but with the oxygens not directly connected to the benzene ring, were negligibly active as inhibitors of BAO. This confirmed the importance of conjugative effects of properly positioned oxygen atoms, which, if far from benzene ring, represented only slightly hydrophilic segments of the substituent chains.

3. Conclusions

With benzylamines **6**, **7**, **8**, (Table 1) we synthesized reversible inhibitors of BAO, very active and selective toward DAO, LO and MAO B. Their significant toxicity, characterized by convulsive effects on treated animals before death, increased with the hydrophobic character of the inhibitor, evidencing their ability to cross the brain barrier.

With benzylamines **10**, **11**, **12**, we synthesized reversible inhibitors of BAO, poorly toxic, up to very active and selective toward DAO, LO and MAO B, and very attractive for the possibility of anchoring inhibitor residues to polymers or resins through the OH groups preserving the inhibitory activity.

With benzylamines **13**, **14**, **15**, endowed with steric, hydrophobic, but not conjugative effects, we synthesized moderately active



Scheme 9. Synthesis of inhibitors **16** and **17**.

Table 1
IC_{50}(mol/L) values of the inhibitory activity of benzylamines **1–17** towards different amine oxidases

Benzylamine	BAO*	DAO#	LO†	MAO B‡	LD_{50} (mg/kg)
1 R_1 = H, R_2 = OH	$>1.0 \times 10^{-3}$ ws	$>1.0 \times 10^{-3}$ ws	$>1.0 \times 10^{-3}$	$>1.0 \times 10^{-3}$ ns	–
2 R_1 = H, R_2 = OCH_2CH_3	$>1.0 \times 10^{-3}$ ws	$>1.0 \times 10^{-3}$ ws	$>1.0 \times 10^{-3}$	$>1.0 \times 10^{-3}$ ws	–
3 R_1 = H, R_2 = CH_2OH	$>1.0 \times 10^{-3}$ ws	$>1.0 \times 10^{-3}$ ws	$>1.0 \times 10^{-3}$	$>1.0 \times 10^{-3}$ ns	–
4 R_1 = R_2 = OH	$>1.0 \times 10^{-3}$ ns	$>1.0 \times 10^{-3}$	$>1.0 \times 10^{-3}$	$>1.0 \times 10^{-3}$	–
5 R_1 = R_2 = OCH_3	$1.2 \pm 0.2 \times 10^{-4}$ r, m, ns	$>1.0 \times 10^{-3}$ ws	$>1.0 \times 10^{-3}$	1.9×10^{-4} ns	120 ± 8
6 R_1 = R_2 = OCH_2CH_3	$1.8 \pm 0.8 \times 10^{-7}$ pr, m, ns	$>1.0 \times 10^{-3}$ ws	$>1.0 \times 10^{-3}$	$>1.0 \times 10^{-3}$ ns	74 ± 20
7 R_1 = R_2 = $O(CH_2)_2CH_3$	$2.4 \pm 0.6 \times 10^{-7}$ pr, m, ns	$>1.0 \times 10^{-3}$ ws	$>1.0 \times 10^{-3}$	$>1.0 \times 10^{-3}$ s	37 ± 5
8 R_1 = R_2 = $O(CH_2)_3CH_3$	$1.4 \pm 0.01 \times 10^{-7}$ pr, nc, ns	$>1.0 \times 10^{-3}$ s	$>1.0 \times 10^{-3}$	$>1.0 \times 10^{-3}$ ns	25 ± 5
9 R_1 = R_2 = $OCH(CH_3)_2$	$>1.0 \times 10^{-3}$ws	$>1.0 \times 10^{-3}$ ws	$>1.0 \times 10^{-3}$	$>1.0 \times 10^{-3}$ ws	–
10 R_1 = R_2 = $O(CH_2)_2OH$	$2.6 \pm 0.2 \times 10^{-6}$ pr, m, ns	$>1.0 \times 10^{-3}$ ws	$>1.0 \times 10^{-3}$ ws	$>1.0 \times 10^{-3}$ ns	>1000
11 R_1 = R_2 = $O(CH_2)_3OH$	$5.5 \pm 1.5 \times 10^{-7}$ pr, m, ns	$>1.0 \times 10^{-3}$ ws	$>1.0 \times 10^{-3}$ ws	$>1.0 \times 10^{-3}$ ws	690 ± 108
12 R_1 = R_2 = $O(CH_2)_4OH$	$6.6 \pm 0.6 \times 10^{-8}$ pr, m, ws	$>1.0 \times 10^{-3}$ ns	$>1.0 \times 10^{-3}$ ws	$>1.0 \times 10^{-3}$ ns	540 ± 305
13 R_1 = R_2 = CH_2CH_3	$4.3 \pm 0.4 \times 10^{-4}$ r, m, ns	$>1.0 \times 10^{-3}$ s	$>1.0 \times 10^{-3}$	$>1.0 \times 10^{-3}$ s	92 ± 11
14 R_1 = R_2 = $(CH_2)_2CH_3$	$1.5 \pm 0.2 \times 10^{-5}$ r, m, ns	$>1.0 \times 10^{-3}$ ws	$>1.0 \times 10^{-3}$	$>1.0 \times 10^{-3}$ ws	55 ± 22
15 R_1 = R_2 = $(CH_2)_3CH_3$	$1.4 \pm 0.3 \times 10^{-5}$ r, m, ns	$>1.0 \times 10^{-3}$ ns	$>1.0 \times 10^{-3}$ ws	$>1.0 \times 10^{-3}$ ns	54 ± 20
16 R_1 = R_2 = CH_2OCH_3	$>1.0 \times 10^{-3}$ r, m, ns	$>1.0 \times 10^{-3}$ ws	$>1.0 \times 10^{-3}$	$>1.0 \times 10^{-3}$ ns	240 ± 24
17 R_1 = R_2 = $CH_2OCH_2CH_3$	$>1.0 \times 10^{-3}$ ns	$>1.0 \times 10^{-3}$ ns	$>1.0 \times 10^{-3}$ ws	$>1.0 \times 10^{-3}$ ws	–

Used enzymes and best substrates:*porcine serum benzylamine oxidase, benzylamine; #porcine kidney diamine oxidase, putrescine; †porcine aorta lysyl oxidase, protein-bound lysine; ‡rat liver monoamine oxidase, 2-phenylethylamine. pr = partially reversible, restored enzyme activity between 50% and 80%; r = reversible, restored enzyme activity over 80%; nc = non competitive; m = mixed; s = substrate; ws = weak substrate; ns = non substrate.

inhibitors of BAO reversible and selective toward DAO, LO and MAO B.

This work and the previous one,[21] both aimed at the discovery of reversible substrate-like inhibitors of CAOs able to form stable complexes with the protein through non-covalent interactions, allowed us to find that CAOs having benzylamine as the preferred substrate can be reversibly inhibited by two different classes of compounds: one correlated to benzylamine (present work) and the other correlated to 4-aminomethylpyridine (previous work) having a pyridine ring capable of coordinative bonds that the benzene ring cannot set up.

In spite of an expected significant diversity in forming non-covalent interactions with the protein, the present work evidences that benzylamine-based inhibitors as those pyridine-based give rise to reversible selective inhibitors with IC_{50} in the order of 10^{-7} mol/L.

In all likelihood, our selective, very active, site-directed, reversible inhibitors of BAO exert their bioactive interactions with the protein by forming, in virtue of their structures and distribution of chemical functions, a plurality of non-covalent bonds, whose knowledge could turn useful for a future inhibitor design.

On such basis the main features of the prepared inhibitors qualify as a set of research tools useful for general biological studies and the formation of enzyme complexes with CAOs for X-ray structure determinations.

The hope of obtaining new knowledge on the substrate specificity of the enzyme from a comparative study of X-ray structures of complexes obtained from a CAO with different inhibitors, finds a significant support in the work of Murakawa et al.[14] where X-ray crystal structures of AGAO complexed with three irreversible inhibitors are determined and compared. By such a way, accounting for unknown naturally broad substrate specificities of the various CAOs and deleterious side effects observed with irreversible inhibitors,[7,15–19] the expectation of attaining useful suggestions for the design of a new generation of reversible inhibitors is founded.

4. Experimental section

4.1. Chemical compounds and methods

Melting points, determined on a Leica Thermogalen III hot stage apparatus, and boiling points are uncorrected. FTIR spectra were recorded as films or KBr pellets on a Perkin Elmer System 2000 spectrophotometer. ^1H and ^{13}C NMR spectra were acquired on a Bruker Avance DPX 300 Spectrometer at 300 and 75.5 MHz, respectively, using TMS as internal standard.

High-resolution mass spectra were obtained by using ESI-QTOF Agilent 6540 spectrometer interfaced with HPLC Agilent 1290 system.

Flash-chromatographic separations were performed on Merck silica gel (0.040–0.063 mm). TLCs were obtained on Merck F_{254} silica gel aluminum sheets. PLCs were performed on Merck F_{254} 60 silica gel plates (20 × 20 cm, 0.5 mm).

All commercial reagents and products were from Sigma–Aldrich and were purified by standard procedures. Petroleum ether refers to the 40–60 °C fraction.

2,6-Dimethoxybenzylamine[24] and its hydrochloride (**5**) were obtained from commercial 2,6-dimethoxybenzonitrile. AlH_3,[26] 2,6-diethylbenzonitrile,[29] 2,6-dipropylbenzaldehyde,[30] 2-(2,6-difluorophenyl)-4,5-dihydro-4,4-dimethyl-1,3-oxazole,[31] 2,6-bis(bromomethyl)benzonitrile,[32] 2-(2-bromoethoxy)tetrahydropyran,[34] 2-(3-bromopropoxy)tetrahydropyran,[35] and 2-(4-bromobutoxy)tetrahydropyran[36] were prepared according to known procedures.

2-Hydroxybenzylamine[37] and 2-ethoxybenzylamine[38] were converted into the corresponding hydrochlorides **1** and **2** by dissolution in dry Et_2O and saturation with dry gaseous HCl. 2-Hydroxymethylbenzylamine hydrochloride (**3**) was obtained from 2-hydroxymethylbenzamide[39] after reduction with lithium aluminum hydride[40] and treatment with dry gaseous HCl as described above.

The 1,3-dialkoxybenzenes **18–21** were prepared by making react 1,3-benzenediol with NaH (as 60% by weight dispersion in mineral oil) in DMF followed by the appropriate alkyl halide, in analogy to the synthesis of 1,3-bis(methoxymethoxy)benzene.[41] 1,3-Diethoxybenzene (**18**): from bromoethane, 78%, bp 105–107 °C/4 torr (Lit.:[42] 234–235 °C). 1,3-Dipropoxybenzene (**19**): from 1-bromopropane, 66%, bp 85–87 °C/0.3 torr (Lit.[43]: 127–128 °C/12 torr). 1,3-Bis(1-methylethoxy)benzene (**20**): from 2-bromopropane, 44%, bp 103–104 °C/4 torr (Lit.[44]: 237–238 °C). 1,3-Dibutoxybenzene (**21**): from 1-bromobutane, 73%, bp 106–109 °C/0.3 torr (Lit.[45]: 290 °C).

4.1.1. 2,6-Dihydroxybenzylamine hydrochloride (4)

2,6-Dimethoxybenzylamine[24] (1.60 g, 9.6 mmol) was treated with 48% aqueous HBr (10 mL) at reflux for 3 h. After removal of the solvent at reduced pressure, the residue was in the order treated with concd HCl (10 mL) and brought to dryness three times up to the disappearance of bromide ion to afford **4** (1.46 g, yield 87%). Mp 203–205 °C (ethanol/Et_2O). IR (KBr, cm^{-1}) 3211, 3045 (NH+OH), 1362 (C–O), 785, 687 (ring). ^1H NMR (DMSO-d_6) δ: 10.03 (s, 2H), 7.96 (br s, 3H), 6.97 (t, 1H, J = 8.1 Hz), 6.43 (d, 2H, J = 8.1 Hz), 3.89 (br q, 2H, J = 3.8 Hz). ^{13}C NMR (DMSO-d_6) δ: 156.91, 129.62, 106.79, 106.13, 31.78. $C_7H_{10}ClNO_2$ requires: C 47.88; H 5.74; N 7.98. Found: C 47.72; H 5.84; N 7.94.

4.1.2. 2,6-Dialkoxybenzaldehydes 22–25

A solution of 1,3-dialkoxybenzene (5.2 mmol) in dry THF (31 mL) was cooled to 0 °C and treated with a 1.66 M solution of butyllithium in hexane (6.2 mmol). The mixture was left under magnetic stirring at room temperature for 2 h, added with dry DMF (1.0 mL, 12.9 mmol), left under stirring for additional 2 h and finally hydrolyzed with 0.5 M HCl (50 mL). The two phases were stirred for 30 min then separated. The aqueous phase was extracted with Et_2O (3 × 20 mL) and the extracts were combined with the organic phase and dried (Na_2SO_4). After removal of the solvent at reduced pressure the crude benzaldehydes were obtained and purified as described below.

4.1.2.1. 2,6-Diethoxybenzaldehyde (22). Yield 74%. Crystallization from hexane. Mp 56–58 °C. IR (KBr, cm^{-1}) 2792, 1682 (CHO), 1249, 1118 (C–O), 779, 721 (ring). ^1H NMR ($CDCl_3$) δ: 10.54 (s, 1H), 7.39 (t, 1H, J = 8.5 Hz), 6.54 (d, 2H, J = 8.5 Hz), 4.11 (q, 4H, J = 7.0 Hz), 1.45 (t, 6H, J = 7.0 Hz). ^{13}C NMR ($CDCl_3$) δ: 189.61, 161.52, 135.61, 114.73, 104.63, 64.54, 14.65. $C_{11}H_{14}O_3$ requires: C 68.02; H 7.27. Found: C 67.98; H 6.98.

4.1.2.2. 2,6-Dipropoxybenzaldehyde (23). Yield 80%. Flash chromatography using a mixture petroleum ether/EtOAc 10/1 as eluent. Oil. IR (film, cm^{-1}): 2772, 1688 (CHO), 1254, 1114 (C–O), 779, 732 (ring). ^1H NMR ($CDCl_3$) δ: 10.56 (s, 1H), 7.38 (t, 1H, J = 8.5 Hz), 6.53 (d, 2H, J = 8.5 Hz), 3.99 (t, 4H, J = 6.5 Hz), 1.91–1.79 (m, 4H), 1.05 (t, 6H, J = 7.4 Hz). ^{13}C NMR ($CDCl_3$) δ: 189.33, 161.69, 135.54, 114.89, 104.61, 70.43, 22.47, 10.53.

HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{13}H_{19}O_3$: 223.1334; found: 223.1327.

4.1.2.3. 2,6-Dibutoxybenzaldehyde (24)[25]. Yield 84%. Flash chromatography using a mixture petroleum ether/EtOAc 15/1 as eluent. Oil. IR (film, cm^{-1}): 2772, 1689 (CHO), 1251, 1105 (C–O), 778, 732 (ring). ^1H NMR ($CDCl_3$) δ: 10.54 (s, 1H), 7.38 (t, 1H, J = 8.4 Hz), 6.53 (d, 2H, J = 8.4 Hz), 4.03 (t, 4H, J = 6.5 Hz), 1.87–1.76 (m, 4H), 1.58–1.44 (m, 4H), 0.97 (t, 6H, J = 7.4 Hz). ^{13}C NMR ($CDCl_3$) δ: 189.37, 161.70, 135.54, 114.82, 104.54, 68.63, 31.12, 19.23, 13.82.

4.1.2.4. 2,6-Bis(1-methylethoxy)benzaldehyde (25)[25]. Yield 72%. Flash chromatography using a mixture petroleum ether/EtOAc 15/1 as eluent. Oil. IR (film, cm^{-1}): 2774, 1688 (CHO), 1252, 1113 (C–O), 780, 722 (ring). ^1H NMR ($CDCl_3$) δ: 10.49 (s, 1H), 7.35 (t, 1H, J = 8.4 Hz), 6.53 (d, 2H, J = 8.4 Hz), 4.62 (sept, 2H, J = 6.1 Hz), 1.37 (d, 12H, J = 6.1 Hz). ^{13}C NMR ($CDCl_3$) δ: 189.77, 160.76; 135.03, 116.88, 106.32, 71.57, 22.04.

4.1.3. Oximes 22a–25a

A solution of 2,6-dialkoxybenzaldehyde (4 mmol) in absolute ethanol (7 mL) was added with pyridine (0.7 mL), then with hydroxylamine hydrochloride (1.11 g, 16 mmol) and heated for 1 h at reflux under magnetic stirring. The mixture was poured into iced water (150 mL) to precipitate the oxime initially as oil which rapidly turned to crystals. After 2 h the oxime was filtered, washed with water, dried at reduced pressure and crystallized.

4.1.3.1. 2,6-Diethoxybenzaldehyde oxime (22a). Yield 90%. Mp 160–162 °C (from benzene, white crystals). ^1H NMR ($CDCl_3$) δ: 10.96 (s, 1H), 8.18 (s, 1H, OH), 7.24 (t, 1H, J = 8.4 Hz), 6.65 (d, 2H, J = 8.4 Hz), 4.04 (q, 4H, J = 7.0 Hz), 1.31 (t, 6H, J = 7.0 Hz). $C_{11}H_{15}NO_3$ requires: C 63.14; H 7.23; N 6.69. Found: C 63.44; H 7.40; N 6.73.

4.1.3.2. 2,6-Dipropoxybenzaldehyde oxime (23a). Yield 89%. Mp 111–113 °C (from benzene/hexane, white crystals). ^1H NMR ($CDCl_3$) δ: 10.25 (s, 1H), 8.55 (s, 1H, OH), 7.20 (t, 1H, J = 8.4 Hz), 6.54 (d, 2H, J = 8.4 Hz), 4.00 (t, 4H, J = 6.7 Hz), 1.90–1.78 (m, 4H), 1.02 (t, 6H, J = 7.4 Hz). $C_{13}H_{19}NO_3$ requires: C 65.80; H 8.07; N 5.90. Found: C 66.13; H 8.30; N 6.05.

4.1.3.3. 2,6-Dibutoxybenzaldehyde oxime (24a). Yield 74%. Mp 90–91 °C (from benzene/hexane, white crystals). ^1H NMR ($CDCl_3$) δ: 9.97 (s, 1H), 8.53 (s, 1H, OH), 7.20 (t, 1H, J = 8.4 Hz), 6.55 (d, 2H, J = 8.4 Hz), 4.04 (t, 4H, J = 6.6 Hz), 1.85–1.76 (m, 4H), 1.59–1.42 (m, 4H), 0.96 (t, 6H, J = 7.4 Hz). $C_{15}H_{23}NO_3$ requires: C 67.90; H 8.74; N 5.28. Found: C 68.17; H 8.94; N 5.16.

4.1.3.4. 2,6-Bis(1-methyethoxy)benzaldehyde oxime (25a). Yield 87%. Mp 153–155 °C (from benzene/hexane, white crystals). ^1H NMR ($CDCl_3$) δ: 10.03 (s, 1H), 8.48 (s, 1H, OH), 7.18 (t, 1H, J = 8.4 Hz), 6.54 (d, 2H, J = 8.4 Hz), 4.57 (sept, 2H, J = 6.1 Hz), 1.37 (d, 12H, J = 6.1 Hz). $C_{13}H_{19}NO_3$ requires: C 65.80; H 8.07; N 5.90. Found: C 66.16; H 8.40; N 5.88.

4.1.4. 2,6-Dialkoxybenzylamine hydrochlorides 6–9

A solution of oxime (2.7 mmol) in ethanol (8.3 mL) was added with a 2 M solution of NaOH (8.3 mL) and treated in small amounts with Nickel Raney alloy (0.65 g) under magnetic stirring. At the end of the hydrogen evolution, the mixture was stirred for 1 h and filtered. The black solid was washed with ethanol (5 × 10 mL) and the washings were combined with the filtrate and concentrated at reduced pressure and 60 °C. The residue was taken with water (3 mL), brought to pH <1 with concd HCl and concentrated at reduced pressure and 60 °C to remove most of the volatiles. The residue was dissolved in water (5 mL), brought to pH >12 with a 2 M solution of NaOH and extracted with Et_2O (3 × 20 mL). After drying over solid NaOH, the extracts were concentrated at reduced pressure to afford the crude amine which was dissolved in dry Et_2O (15 mL), treated with a 1.2 M solution of gaseous HCl in Et_2O until pH strongly acid to precipitate the hydrochloride which was filtered, dried over P_2O_5 and crystallized.

4.1.4.1. 2,6-Diethoxybenzylamine hydrochloride (6). Yield 80%. Mp 199–202 °C (from acetonitrile, white crystals). IR (KBr, cm^{-1}): 2980, 2931 (NH+CH), 1258, 1134 (C–O), 775, 724 (ring).

^{1}H NMR (DMSO-d_6) δ: 8.22 (br s, 3H), 7.30 (t, 1H, J = 8.4 Hz), 6.67 (d, 1H, J = 8.4 Hz), 4.07 (q, 4H, J = 7.0 Hz), 3.93 (s, 2H), 1.37 (t, 6H, J = 7.0 Hz). ^{13}C NMR (DMSO-d_6) δ: 158.12, 131.07, 109.88, 104.98, 64.34, 31.65, 15.03. $C_{11}H_{18}ClNO_2$ requires: C 57.02; H 7.83; N 6.04; Cl 15.30. Found: C 57.15; H 7.82; N 6.08; Cl 15.35.

4.1.4.2. 2,6-Dipropoxybenzylamine hydrochloride (7). Yield 25%. Mp 145–147 °C (from acetonitrile, white crystals). IR (KBr, cm^{-1}): 2963, 2937, 2906 (NH+CH), 1259, 1134 (C–O) 778, 731 (ring). ^{1}H NMR (DMSO-d_6) δ: 8.18 (br s, 3H); 7.30 (t, 1H, J = 8.4 Hz); 6.67 (d, 2H, J = 8.4 Hz); 3.97 (t, 4H, J = 6.5 Hz); 3.94 (s, 2H); 1.85–1.73 (m, 4H), 1.00 (t, 6H, J = 7.3 Hz). ^{13}C NMR (DMSO-d_6) δ: 157.68, 130.55, 109.41, 104.44, 69.56, 31.08, 21.85, 10.37. $C_{13}H_{22}ClNO_2$ requires: C 60.11; H 8.54; N 5.39; Cl 13.65. Found: C 60.48; H 8.60; N 5.42; Cl 13.57.

4.1.4.3. 2,6-Dibutoxybenzylamine hydrochloride (8). Yield 16%. Mp 147–149 °C (from Et_2O/hexane, white solid). IR (KBr, cm^{-1}): 2960, 2873 (NH+CH), 1255, 1127 (C–O), 775, 722 (ring). ^{1}H NMR (DMSO-d_6) δ: 8.14 (br s, 3H), 7.30 (t, 1H, J = 8.4 Hz), 6.68 (d, 2H, J = 8.4 Hz), 4.01 (t, 4H, J = 6.5 Hz), 3.93 (s, 2H), 1.80–1.71 (m, 4H), 1.52–1.39 (m, 4H), 0.94 (t, 6H, J = 7.4 Hz). ^{13}C NMR (DMSO-d_6) δ: 157.69, 130.54, 109.37, 104.42, 67.80, 31.12, 30.58, 18.64, 13.70. $C_{15}H_{26}ClNO_2$ requires: C 62.59; H 9.10; N 4.87; Cl 12.32. Found: C 62.88; H 9.25; N 4.87; Cl 12.45.

4.1.4.4. 2,6-Bis(1-methylethoxy)benzylamine hydrochloride (9). Yield 53%. Mp 132–134 °C (from EtOAc, white needles). IR (KBr, cm^{-1}): 2978, 2935, 2873 (NH+CH), 1260, 1126 (C–O), 781, 729 (ring). ^{1}H NMR (DMSO-d_6) δ: 8.10 (br s, 3H), 7.29 (t, 1H, J = 8.4 Hz), 6.66 (d, 2H, J = 8.4 Hz), 4.63 (sept, 2H, J = 6.0 Hz), 3.90 (s, 2H), 1.31 (d, J = 6.0 Hz, 12H). ^{13}C NMR (DMSO-d_6) δ: 156.79, 130.31, 110.80, 105.28, 70.17, 31.38, 21.70. $C_{13}H_{22}ClNO_2$ requires: C 60.11; H 8.54; N 5.39; Cl 13.65. Found: C 60.23; H 8.61; N 5.36; Cl 13.40.

4.1.5. 2,2′-(1,3-Phenylenedioxy)bisethanol, tetrahydropyranyl ether (26)

NaH (3.18 g, 79.5 mmol as 60% by weight dispersion in mineral oil) was washed under nitrogen and stirring with dry petroleum ether (3 × 20 mL) and suspended in dry DMF (25 mL). The suspension was treated with a solution of 1,3-benzenediol (4.00 g, 36.3 mmol) in dry DMF (15 mL) in 15 min, left under stirring at room temperature for 3 h and added with 2-(2-bromoethoxy)tetrahydropyran[34] (15.03 g, 71.9 mmol). The mixture was left overnight at room temperature, hydrolyzed with water (150 mL) and extracted with Et_2O (3 × 30 mL). The extracts were washed twice with a 10% NaOH solution, dried (Na_2SO_4), concentrated at reduced pressure and freed from the volatiles at 120 °C/0.1 torr. The residue was filtered through a short basic alumina plug (15 g) using pentane (150 mL) as eluent affording after removal of pentane at reduced pressure **26** as a clear oil (7.95 g, 60%). For characterization purposes a sample of **26** was chromatographed (PLC) using a mixture of petroleum ether/ethyl acetate = 3/1 as eluent. IR (KBr, cm^{-1}) 1184, 1158, 1141, 1126, 1079, 1036 (tetrahydropyranyl ring). ^{1}H NMR ($CDCl_3$) δ: 7.19–7.11 (m, 1H), 6.56–6.51 (m, 3H), 4.74–4.67 (m, 2H), 4.19–3.77 (m, 10H), 3.58–3.46 (m, 2H), 1.93–1.47 (m, 12H). ^{13}C NMR ($CDCl_3$) δ: 160.10, 129.77, 107.14, 101.98, 98.97, 67.42, 65.80, 62.18, 30.52, 25.43, 19.36. $C_{20}H_{30}O_6$ requires: C 65.55; H 8.25. Found: C 65.64; H 8.01.

4.1.6. 3,3′-(1,3-Phenylenedioxy)bispropanol, tetrahydropyranyl ether (27)

Following a similar procedure, **27** was synthesized from 1,3-benzenediol and 2-(3-bromopropoxy)tetrahydropyran.[35] Clear oil. Yield 72%. IR (KBr, cm^{-1}) 1183, 1155, 1140, 1124, 1077, 1035 (tetrahydropyranyl ring). ^{1}H NMR ($CDCl_3$) δ: 7.18–7.11 (m, 1H), 6.53–6.46 (m, 3H), 4.63–4.57 (m, 2H), 4.12–4.02 (m, 4H), 3.96–3.80 (m, 4H), 3.61–3.45 (m, 4H), 2.07 (quintet, 4H, J = 6.5 Hz), 1.88–1.46 (m, 4H). ^{13}C NMR ($CDCl_3$) δ: 160.25, 129.76, 106.78, 101.55, 98.94, 64.92, 64.03, 62.30, 30.70, 29.70, 25.47, 19.59. $C_{22}H_{34}O_6$ requires: C 66.98; H 8.69. Found: C 66.96; H 8.89.

4.1.7. 4,4′-(1,3-Phenylenedioxy)bisbutanol, tetrahydropyranyl ether (28)

Following a similar procedure, **28** was synthesized from 1,3-benzenediol and 2-(4-bromobutoxy)tetrahydropyran.[36] Clear oil. Yield 80%. IR (KBr, cm^{-1}) 1255, 1130, 1030 (tetrahydropyranyl ring). ^{1}H NMR ($CDCl_3$) δ: 7.17–7.11 (m, 1H), 6.50–6.45 (m, 3H), 4.60 (t, 2H, J = 2.7 Hz), 3.97 (t, 4H, J = 6.3 Hz), 3.91–3.77 (m, 4H), 3.54–3.41 (m, 4H), 1.92–1.52 (m, 20H). ^{13}C NMR ($CDCl_3$) δ: 160.27, 129.76, 106.63, 101.38, 98.83, 67.63, 67.11, 62.31, 30.73, 26.35, 26.21, 25.48, 19.62. $C_{24}H_{38}O_6$ requires: C 68.22; H 9.06. Found: C 68.49; H 9.34.

4.1.8. 2,6-Bis(2-hydroxyethoxy)benzaldehyde (29)[27]

A solution of bis(tetrahydropyranyl) ether **26** (4.04 g, 11.0 mmol) in dry Et_2O (15 mL) was treated at 0 °C under nitrogen and stirring with a 1.66 M solution of butyllithium in hexane (7.6 mL, 12.6 mmol). The mixture was refluxed for 4 h, cooled to 0 °C, treated with dry DMF (1.25 mL, 16.2 mmol), left overnight under stirring at room temperature and hydrolyzed with water (20 mL). The aqueous phase was separated, extracted with Et_2O (2 × 20 mL) and combined with the organic phase. All the extracts were washed twice with a 5% solution of NaOH and dried (Na_2SO_4). The removal of the solvent at reduced pressure afforded a residue which was purified by Flash Chromatography (FC) using a mixture of petroleum ether/acetone 6/1 as eluent to yield the bistetrahydropyranyl derivative of **29** as a viscous oil (2.44 g, yield 56%). Spectral data coincident with those known.[27]

The oil was taken with MeOH (18 mL), diluted with water (8 mL), treated with concd HCl (4 mL) and left at room temperature for 3 h. The clear solution was neutralized with solid K_2CO_3 and extracted with EtOAc (8 × 15 mL). The extracts were washed with water, dried (Na_2SO_4) and concentrated at reduced pressure to afford **29** as oil which was crystallized from EtOAc/Et_2O at −18 °C (0.702 g, total yield 28%). Mp 101–103 °C (from EtOAc, white leaflets, Lit.[27] 101–103 °C from EtOAc). IR (KBr, cm^{-1}) 3431, 3331 (OH), 2964, 2814 (CH_2), 1678 (CH=O), 1253, 1113 (C–O), 784, 726 (ring). ^{1}H NMR ($CDCl_3$) δ: 10.48 (s, 1H), 7.41 (t, 1H, J = 8.5 Hz), 6.56 (d, 2H, J = 8.5 Hz), 4.17–4.11 (m, 4H), 4.01–3.95 (m, 4H), 4.33–3.87 (very broad, 2H). ^{13}C NMR ($CDCl_3$) δ: 190.46, 161.63, 136.38, 114.95, 105.80, 71.08, 60.88. $C_{11}H_{14}O_5$ requires: C 58.40; H 6.24. Found: C 58.23; H 6.44.

4.1.9. 2,6-Bis(3-hydroxypropoxy)benzaldehyde (30)

Benzaldehyde **30** was prepared from **27** as described above with the difference that the bistetrahydropyranyl derivative of **30** was not submitted to FC but directly deprotected through acid hydrolysis to afford crude **30** which was purified by FC using a mixture of petroleum ether/acetone 2/1 followed by petroleum ether/acetone 1.5/1 as eluents. Yield 43%. Mp 73–75 °C (from EtOAc, white crystals). IR (KBr, cm^{-1}) 3396, 3208 (OH), 2944, 2782 (CH_2), 1678 (CH=O), 1253, 1114 (C–O), 776, 719 (ring). ^{1}H NMR ($CDCl_3$) δ: 10.46 (s, 1H), 7.43 (t, 1H, J = 8.5 Hz), 6.56 (d, 2H, J = 8.5 Hz), 4.18 (t, 4H, J = 5.9 Hz), 3.86 (t, 4H, J = 5.5 Hz), 3.68 (br s, 2H), 2.14–2.03 (m, 4H). ^{13}C NMR ($CDCl_3$) δ: 189.61, 161.64, 136.55, 113.79, 104.45, 67.46, 60.45, 31.84. $C_{13}H_{18}O_5$ requires: C 61.40; H 7.13. Found: C 61.26; H 7.27.

4.1.10. 2,6-Bis(4-hydroxybutoxy)benzaldehyde (31)

Benzaldehyde **31** was prepared as described above after direct deprotection through acid hydrolysis of the product of lithiation/

formylation of **28**. Crude **31** was purified by FC using a mixture of petroleum ether/acetone 2/3 followed by petroleum ether/acetone 1/4 as eluents. Yield 43%. Mp 79–80 °C (from EtOAc, white crystals). IR (KBr, cm^{-1}) 3443 (OH), 2954, 2875 (CH_2), 1675 (CH=O), 1252, 1106 (C–O), 787 (ring). ^1H NMR ($CDCl_3$) δ: 10.48 (s, 1H), 7.40 (t, 1H, J = 8.5 Hz), 6.54 (d, 2H, J = 8.5 Hz), 4.07 (t, 4H, J = 5.9 Hz), 3.71 (t, 4H, J = 6.2 Hz), 2.93 (br s, 2H), 1.99–1.91 (m, 4H), 1.80–1.71 (m, 4H). ^{13}C NMR ($CDCl_3$) δ: 189.68, 161.71, 136.22, 114.24, 104.53, 68.98, 61.78, 29.72, 25.31. $C_{15}H_{22}O_5$ requires: C 63.81; H 7.85. Found: C 63.44; H 7.90.

4.1.11. 2,6-Bis(ω-hydroxyalkoxy)benzylamine hydrochlorides 10–12

A solution of benzaldehyde **29**–**31** (40 mmol) in MeOH (7 mL) was treated in a 125 mL autoclave with a 6.6 N ammonia solution in MeOH (19 mL) at 80 °C and 80 atm of hydrogen pressure for 7 h in the presence of Ni Raney (1.60 g). After filtration, the catalyst was washed with MeOH, the washings were combined with the filtrate and concentrated at reduced pressure. The crude benzylamine derivatives were dissolved in dry THF (200 mL) and treated with gaseous HCl up to complete precipitation of the hydrochlorides which were filtered, washed with THF and dried in vacuo.

4.1.11.1. 2,6-Bis(2-hydroxyethoxy)benzylamine hydrochloride (10). Yield 78%. Mp 166–168 °C (from acetonitrile/ethanol, white crystals). IR (KBr, cm^{-1}) 3293 (OH), 2962, 2939 (NH+CH), 1260, 1138 (C–O). ^1H NMR (DMSO-d_6) δ: 8.15 (br s, 3H), 7.31 (t, 1H; J = 8.4 Hz), 6.70 (d, 2H, J = 8.4 Hz), 5.17 (br s, 2H), 4.08–4.00 (m, 6H), 3.75 (br s, 4H). ^{13}C NMR (DMSO-d_6) δ: 157.70, 130.52, 110.02, 105.08, 70.41, 59.32, 31.31. $C_{11}H_{18}ClNO_4$ requires: C 50.10; H 6.88; N 5.31. Found C 49.89; H 6.87; N 5.31.

4.1.11.2. 2,6-Bis(3-hydroxypropoxy)benzylamine hydrochloride (11). Yield 78%. Mp 156–158 °C (from ethanol/acetonitrile 1/3, white crystals). IR (KBr, cm^{-1}) 3325 (OH), 2947, 2886 (NH+CH), 1259, 1126 (C–O), 773, 737 (ring). ^1H NMR (DMSO-d_6) δ: 8.12 (br s, 3H), 7.31 (t, 1H, J = 8.4 Hz), 6.69 (d, 2H, J = 8.4 Hz), 4.75 (br t, 2H, J = 4.7 Hz), 4.09 (t, 4H, J = 6.2 Hz), 3.95 (s, 2H), 3.64-3.56 (m, 4H), 1.91 (quintet, 4H, J = 6.1 Hz). ^{13}C NMR (DMSO-d_6) δ: 157.62, 130.55, 109.32, 104.41, 65.52, 57.46, 31.75, 31.28. $C_{13}H_{22}ClNO_4$ requires: C 53.51; H 7.60; N 4.80. Found: C 53.12; H 7.68; N 4.35.

4.1.11.3. 2,6-Bis(4-hydroxybutoxy)benzylamine hydrochloride (12). Yield 78%. Mp 126–128 °C (from acetonitrile, white crystals). IR (KBr, cm^{-1}) 3412 (OH), 2949, 2872 (NH+CH), 1257, 1126, (C–O), 778, 726 (ring). ^1H NMR (DMSO-d_6) δ: 8.00 (br s, 3H), 7.30 (t, 1H, J = 8.4 Hz), 6.68 (d, 2H, J = 8.4 Hz), 4.51 (br s, 2H), 4.02 (t, 2H, J = 6.5 Hz), 3.95 (br q, 2H, J = 4.4 Hz), 3.46 (t, 2H, J = 6.4 Hz), 1.85–1.76 (m, 2H), 1.63–1.54 (m, 2H). ^{13}C NMR (DMSO-d_6) δ: 157.64, 130.60, 109.29, 104.47, 68.09, 60.31, 31.26, 28.80, 25.38. $C_{15}H_{26}ClNO_4$ requires: C 56.33; H 8.19; N 4.38. Found: C 56.24; H 8.43; N 4.16.

4.1.12. 2,6-Diethylbenzylamine hydrochloride (13)

A solution of 2,6-diethylbenzonitrile[29] (1.59 g, 10.0 mmol) in dry Et_2O (50 mL) was added dropwise to a suspension of $LiAlH_4$ (0.90 g, 23.7 mol) in dry Et_2O (60 mL). The mixture was refluxed under nitrogen and stirring for 6 h, hydrolyzed with 10% aqueous NaOH (35 mL). The aqueous phase was separated and extracted with fresh Et_2O and the extracts combined with the organic phase were dried over KOH. After removal of the solvent at reduced pressure, the residue was dissolved in Et_2O (50 mL) and saturated with gaseous HCl to precipitate hydrochloride **13** which was dried at reduced pressure and crystallized (1.54 g, yield 77%). Mp 240–243 °C (from acetonitrile, fluffy, white needles). IR (KBr, cm^{-1}) 3034, 2969, 2935 (NH+CH) 804, 761 (ring). ^1H NMR (DMSO-d_6) δ: 8.50 (br s, 3H), 7.32–7.25 (m, 1H), 7.12 (d, 2H, J = 7.6 Hz), 4.01 (s, 2H), 2.74 (q, 4H, J = 7.5 Hz), 1.17 (t, 6H, J = 7.5 Hz). ^{13}C NMR (DMSO-d_6) δ: 143.64, 129.39, 129.05, 126.12, 34.72, 25.18, 15.17. $C_{11}H_{18}ClN$ requires: C 66.15; H 9.08; N 7.01. Found: C 65.84; H 8.99; N 6.79.

4.1.13. 2,6-Dipropylbenzylamine hydrochloride (14)

A solution of 2,6-dipropylbenzaldehyde (**34**)[30] (2.50 g, 13.1 mol) in pyridine (20 mL) was treated with hydroxylamine hydrochloride (2.74 g, 39.4 mol) for 1 h over a boiling water bath. The mixture was hydrolyzed with water (40 mL), treated with concd HCl up to pH = 1 and extracted with Et_2O (5 × 50 mL). The organic phase and extracts were combined, dried (Na_2SO_4) and concentrated at reduced pressure to afford the 2,6-dipropylbenzaldehyde oxime (**36**) (2.13 g, yield 79%). Oil. IR (KBr, cm^{-1}) 3326 (OH), 1632 (w, C=N), 774, 749 (ring). ^1H NMR ($CDCl_3$) δ: 8.40 (s, 1H), 7.85 (br s, 1H), 7.24–7.18 (m, 1H), 7.10–7.04 (m, 2H), 2.72–2.63 (m, 4H), 1.65–1.52 (m, 4H), 0.94 (t, 6H, J = 7.3 Hz). ^{13}C NMR ($CDCl_3$) δ: 149.62, 142.17, 128.81, 127.46, 35.99, 24.40, 14.07 (one aromatic signal is hidden). HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{13}H_{20}NO$: 206.1545; found: 206.1538.

Oxime **36** (2.13 g, 10.4 mmol) was dissolved in dry THF (24 mL) and treated under nitrogen and stirring at reflux for 1 h with a THF solution of AlH_3[26] (29 mL; 0.72 M). The mixture was hydrolyzed with water (2 mL), 10% aqueous NaOH (2 mL), filtered and the filter cake washed with Et_2O (3 × 50 mL). Filtrate and washings were combined and dried over KOH. After removal of the solvent at reduced pressure the oily residue was dissolved in dry Et_2O (40 mL) and saturated with gaseous HCl to afford **14** which was filtered, washed with Et_2O and dried at reduced pressure (1.78 g, yield 75%). Mp 220–222 °C (acetonitrile, white solid). IR (KBr, cm^{-1}) 2961, 2933, 2872 (NH+CH), 789, 771 (ring). ^1H NMR (DMSO-d_6) δ: 8.43 (br s, 3H), 7.28–7.22 (m, 1H), 7.10 (d, 2H, J = 7.6 Hz), 4.00 (s, 2H), 2.67 (t, 4H, J = 7.8 Hz), 1.62–1.48 (m, 4H), 0.96 (t, 6H, J = 7.3 Hz). ^{13}C NMR (DMSO-d_6) δ: 142.24, 129.73, 128.68, 127.08, 34.86, 34.34, 23.98, 13.89. $C_{13}H_{22}ClN$ requires: C 68.55; H 9.74; N 6.15; Cl 15.57. Found: C 68.30; H 10.11; N 5.96; Cl 15.28.

4.1.14. 2,6-Dibutylbenzylamine hydrochloride (15)

A solution of 2,6-dibutylbenzaldehyde (**35**) (Supplementary data) (2.00 g, 9.2 mol) in pyridine (20 mL) was treated with hydroxylamine hydrochloride (1.90 g, 27.3 mol) for 1 h at 60 °C. The mixture was hydrolyzed with water (40 mL), treated with concd HCl up to pH = 1 and extracted with Et_2O (5 × 50 mL). The organic phase and extracts were combined, dried (Na_2SO_4) and concentrated at reduced pressure to afford 2,6-dibutylbenzaldehyde oxime (**37**) (1.85 g, yield 86%). Oil. IR (KBr, cm^{-1}) 3326 (OH), 1635 (w, C=N), 762, 750 (ring). ^1H NMR ($CDCl_3$) δ: 8.65 (br s, 1H), 8.39 (s, 1H), 7.23–7.18 (m, 1H), 7.06 (d, 2H, J = 7.7 Hz), 2.73–2.64 (m, 4H), 1.59–1.47 (m, 4H), 1.41–1.27 (m, 4H), 0.91 (t, 6H, J = 7.3 Hz). ^{13}C NMR ($CDCl_3$) δ: 149.39; 142.37; 128.88; 127.28; 33.56; 33.44; 22.63; 13.95 (one aromatic signal is hidden). HRMS (ESI) m/z: $[M+H]^+$ calcd for $C_{15}H_{24}NO$: 234.1858; found: 234.1850.

Oxime **37** (1.80 g, 7.7 mmol) was dissolved in dry THF (20 mL) and treated under nitrogen and stirring at reflux for 1 h with a THF solution of AlH_3[26] (30 mL; 0.64 M). The mixture was hydrolyzed with water (4 mL), 10% aqueous NaOH (6 mL), filtered and the filter cake washed with Et_2O (3 × 50 mL). Filtrate and washings were combined and dried over KOH. After removal of the solvent at reduced pressure the oily residue was dissolved in dry Et_2O (40 mL) and saturated with gaseous HCl to afford **15** which was filtered, washed with Et_2O and dried at reduced pressure (1.44 g, yield 73%). Mp 146–147 °C (hexane, white solid). IR (KBr, cm^{-1})

2957, 2931, 2872 (NH+CH), 805, 762 (ring). ^{1}H NMR (DMSO-d_6) δ: 8.41 (br s, 3H), 7.27–7.22 (m, 1H), 7.09 (d, 2H, J = 7.6 Hz), 3.99 (s, 2H); 2.72–2.64 (m, 4H), 1.56–1.32 (m, 8H), 0.93 (t, 6H, J = 7.2 Hz). ^{13}C NMR (DMSO-d_6) δ: 142.46, 129.60, 128.75, 127.00, 34.91, 33.10, 32.08, 22.11, 13.77. $C_{15}H_{26}ClN$ requires: C 70.42; H 10.24; N 5.48; Cl 13.86. Found: C 70.18; H 10.48; N 5.17; Cl 14.20.

4.1.15. 2,6-Bis(alkoxymethyl)benzonitriles 38 and 39

Solid 2,6-bis(bromomethyl)benzonitrile[32] (2.00 g, 6.9 mmol) was added to a suspension of sodium methoxide or ethoxide (17.5 mmol) in dry DMF (12 mL) cooled to 0 °C. The dark mixture was stirred at 0 °C for 3 h, diluted with water (20 mL) and extracted with Et_2O (5 × 30 mL). The extracts were washed with water (20 mL), dried (Na_2SO_4), removed from the solvent at reduced pressure to afford crude nitriles **38** and **39** purified as described below.

4.1.15.1. 2,6-Bis(methoxymethyl)benzonitrile (38). Yield 47%. Sublimation at 80 °C/0.01 torr. Mp 80–81 °C (from hexane, white solid). IR (KBr, cm^{-1}) 2220 (CN), 1119 (C–O), 810, 742 (ring). ^{1}H NMR ($CDCl_3$) δ: 7.62–7.47 (m, 3H); 4.66 (s, 4H); 3.48 (s, 6H). ^{13}C NMR ($CDCl_3$) δ: 142.39; 132.70; 127.47; 115.49; 110.32; 72.26; 58.87. $C_{11}H_{13}NO_2$ requires: C 69.09; H 6.85; N 7.32. Found: C 69.37; H 7.15; N 7.14.

4.1.15.2. 2,6-Bis(ethoxymethyl)benzonitrile (39). Yield 37%. Oil. Flash chromatography using a mixture petroleum ether/EtOAc = 10/2 as eluent. IR (KBr, cm^{-1}) 2219 (CN),1130, 1115 (C–O), 792, 733(w) (ring). ^{1}H NMR ($CDCl_3$) δ: 7.61–7.47 (m, 3H), 4.69 (s, 4H), 3.64 (q, 4H, J = 7.0 Hz), 1.28 (t, 6H, J = 7.0 Hz). ^{13}C NMR ($CDCl_3$) δ: 142.76, 132.67, 127.38, 115.56, 110.25, 70.32, 66.71, 15.12. $C_{13}H_{17}NO_2$ requires: C 71.21; H 7.81; N 6.39. Found: C 70.98; H 7.87; N 6.30.

4.1.16. 2,6-Bis(alkoxymethyl)benzylamine hydrochlorides 16 and 17

A solution of nitrile **38** or **39** (2.6 mmol) in dry Et_2O (50 mL) was added in 15 min under N_2 and stirring to a suspension of $LiAlH_4$ (0.40 g, 10.5 mmol) in Et_2O (25 mL). The suspension was refluxed for 5 h, cooled to 0 °C and hydrolyzed with 20% aqueous NaOH (1.5 mL). The solid was filtered and washed with Et_2O (5 × 10 mL). Filtrate and washings were combined and concentrated at reduced pressure to afford an oily residue which was dissolved in dry Et_2O (30 mL) and treated under stirring with 1.2 M gaseous HCl in Et_2O (3 mL) to afford hydrochloride **16** or **17**.

4.1.16.1. 2,6-Bis(methoxymethyl)benzylamine hydrochloride (16). Yield 94%. Mp 167–168 °C (from acetonitrile, white crystals). IR (KBr, cm^{-1}) 3152, 2998, 2935 (NH+CH), 1102, 1080 (C–O), 797, 765 (ring). ^{1}H NMR (DMSO-d_6) δ: 8.28 (br s, 3H), 7.39 (s, 3H), 4.62 (s, 4H), 4.08 (s, 2H), 3.34 (s, 6H). ^{13}C NMR (DMSO-d_6) 138.43, 131.62, 129.38, 128.69, 72.02, 57.68, 34.98. $C_{11}H_{18}ClNO_2$ requires: C 57.02; H 7.83; N 6.04. Found: C 56.80; H 8.11; N 5.88.

4.1.16.2. 2,6-Bis(ethoxymethyl)benzylamine hydrochloride (17). Yield 88%. Mp 129–130 °C (from EtOAc, white needles). IR (KBr, cm^{-1}) 3144, 2970, 2862 (NH+CH), 1089, 1072 (C–O), 800, 767 (ring). ^{1}H NMR (DMSO-d_6) δ: 8.30 (br s, 3H), 7.42–7.34 (m, 3H), 4.66 (s, 4H), 4.11 (s, 2H), 3.56 (q, 4H, J = 7.0 Hz), 1.18 (t, 6H, J = 7.0 Hz). ^{13}C NMR (DMSO-d_6) 138.68, 131.49, 129.20, 128.73, 70.00, 65.28, 35.00, 14.92. $C_{13}H_{22}ClNO_2$ requires: C 60.11; H 8.54; N 5.39. Found: C 60.17; H 8.43; N 5.08.

4.2. Biological materials and methods

Pure BAO from porcine serum was prepared by the method of Buffoni and Blaschko.[46] DAO from porcine kidney (specific activity 100 ± 8.9 nmol mL^{-1} h^{-1}) was obtained from Sigma. LO from porcine aorta was prepared according to Buffoni and Raimondi.[47]

Mitochondria used as source of MAO B (specific activity 12.5 ± 0.7 nmol mL^{-1} min^{-1}) were obtained from rat liver by homogeneization 1:10 in 0.01 M phosphate buffer pH 7.4 containing 0.25 M sucrose and sequential centrifugation at 1000g for 20 min and 10,000g for 20 min.

The enzymatic activity was systematically determined using radioactive labeled substrates as reported by Ignesti et al.[48] The labeled substrates such as [7-^{14}C]benzylamine, 2-phenyl[1-^{14}C]ethylamine, and [1,4-^{14}C]putrescine were obtained from Amersham Biosciences. Tritiated protein-bound lysine was prepared according to Pinnel and Martin[49] with successive modification.[50] The enzymatic activity of MAO-B was assayed using 2-phenyl[1-^{14}C]ethylamine as described by Pino et al.[51] In some cases the enzymatic reaction was followed by measuring the production of H_2O_2 with the method of 4-aminoantipyrine[52] either for the activity of BAO, DAO and LO, or the evaluation of the various compounds as substrates of the same enzymes. The protein content was determined by the method of Lowry et al.[53]

The type of inhibition was evaluated by examining the effect of the inhibitor on the enzymatic reaction velocity (V) at 5 different substrate concentrations (S) and plotting the results with the classical double reciprocal method 1/V versus 1/S proposed by Lineweaver and Burk.[54]

The IC_{50} values for the examined inhibitors were established, as means of 4 determinations, pre-incubating the enzyme for 30 min at 30 °C with the inhibitor at 6 different concentrations, then adding the proper labeled substrate at a saturating concentration, and performing the enzyme activity tests. IC_{50} were estimated from plots of the percentage enzyme activity remaining after inhibition versus negative logarithm of the inhibitor concentration.

Preliminary tests of reversibility were performed to show that the inhibition produced by inhibitor at IC_{50} concentrations was reverted by dialysis, then the reversibility was quantified as follows. From weighted mother solutions, two initial solutions were prepared, a solution containing enzyme and inhibitor at a concentration of 4 × IC_{50} and a control solution where the inhibitor was replaced by an equivalent volume of phosphate buffer pH 7.4. After 5 min at 37 °C the two initial solutions were employed in preparing diluted solutions 1:10 and 1:100 and withdrawing aliquots for activity tests, while the residual portions were dialyzed against water at 4 °C for 24 h. The initial, 1:10 and 1:100 diluted, and dialyzed solutions, with or without inhibitor, were submitted to radiochemical enzyme activity tests using [7-^{14}C]benzylamine as substrate at a saturating concentration, stopping the enzymatic reaction with 3 N HCl, extracting with ethyl acetate and measuring the produced radioactive benzaldheyde. The inhibition reversibility was determined as percentage of activity with respect to a reaction performed under the same conditions without inhibitor.

Tests of acute toxicity (LD_{50}) were performed on *Mus musculus*, Swiss white by intraperitoneal administration of five scaled doses to 10 animals for each dose. The mortality data were elaborated and the standard errors calculated according to Randhawa.[55] The toxic symptomatology began after 15–20 min and was characterized by tonic-clonic convulsions followed by respiratory block. No apparent toxic symptoms were observed for 48 h on survived animals.

Acknowledgment

We thank Ms Jane Erkkila for language help and University of Genoa for financial support (Progetti di Ricerca di Ateneo).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2014.01.037.

References and notes

1. (a) Klinman, J. P. *Chem. Rev.* **1996**, *96*, 2541; (b) Klinman, J. P. *Biochim. Biophys. Acta* **2003**, *1647*, 131.
2. Parsons, M. R.; Convery, M. A.; Wilmot, C. M.; Yadav, K. D. S.; Blakeley, V.; Corner, A. S.; Phillips, S. E. V.; McPherson, M. J.; Knowles, P. F. *Structure* **1995**, *3*, 1171.
3. Wilce, M. C. J.; Dooley, D. M.; Freeman, H. C.; Guss, J. M.; Matsunami, H.; McIntire, W. S.; Ruggiero, C. E.; Tanizawa, K.; Yamaguchi, H. *Biochemistry* **1997**, *36*, 16116.
4. Li, R.; Klinman, J. P.; Mathews, F. S. *Structure* **1998**, *6*, 293.
5. Duff, A. P.; Cohen, A. E.; Ellis, P. J.; Kuchar, J. A.; Langley, D. B.; Shepard, E. M.; Dooley, D. M.; Freeman, H. C.; Guss, J. M. *Biochemistry* **2003**, *42*, 15148.
6. Kumar, V.; Dooley, D. M.; Freeman, H. C.; Guss, J. M.; Harvey, I.; McGuirl, M. A.; Wilce, M. C. J.; Zubak, V. M. *Structure* **1996**, *4*, 943.
7. McGrath, A. P.; Hilmer, K. M.; Collyer, C. A.; Shepard, E. M.; Elmore, B. O.; Brown, D. E.; Dooley, D. M.; Guss, J. M. *Biochemistry* **2009**, *48*, 9810.
8. Wang, S. X.; Mure, M.; Medzihradszky, K. F.; Burlingame, A. L.; Brown, D. E.; Dooley, D. M.; Smith, A. J.; Kagan, H. M.; Klinman, J. P. *Science* **1996**, *273*, 1078.
9. Wilmot, C. M.; Murray, J. M.; Alton, G.; Parsons, M. R.; Convery, M. A.; Blakeley, V.; Corner, A. S.; Palcic, M. M.; Knowles, P. F.; McPherson, M. J.; Phillips, S. E. V. *Biochemistry* **1997**, *36*, 1608.
10. O'Connell, K. M.; Langley, D. B.; Shepard, E. M.; Duff, A. P.; Jeon, H.-B.; Sun, G.; Freeman, H. C.; Guss, J. M.; Sayre, L. M.; Dooley, D. M. *Biochemistry* **2004**, *43*, 10965.
11. Wilmot, C. M.; Saysell, C. G.; Blessington, A.; Conn, D. A.; Kurtis, C. R.; McPherson, M. J.; Knowles, P. F.; Phillips, S. E. V. *FEBS Lett.* **2004**, *576*, 301.
12. Saysell, C. G.; Tambyrajah, W. S.; Murray, J. M.; Wilmot, C. M.; Phillips, S. E. V.; McPherson, M. J.; Knowles, P. F. *Biochem. J.* **2002**, *365*, 809.
13. Ernberg, K.; Zhong, B.; Ko, K.; Miller, L.; Nguyen, Y.; Sayre, L. M.; Guss, J. M.; Lee, I. *Biochim. Biophys. Acta: Proteins Proteomics* **2011**, *1814*, 638.
14. Murakawa, T.; Hayashi, H.; Taki, M.; Yamamoto, Y.; Kawano, Y.; Tanizawa, K.; Okajima, T. *J. Biochem.* **2012**, *151*, 167.
15. O'Brien, J. G.; Dong, B. J.; Coleman, R. L.; Gee, L.; Balano, K. B. *Clin. Infect. Dis.* **1997**, *24*, 854.
16. Balslev, U.; Nielsen, T. L. *Dan. Med. Bull.* **1992**, *39*, 366.
17. Briceland, L. L.; Bailie, G. R. *Ann. Pharmacother.* **1991**, *25*, 1171.
18. Sensakovic, J. W.; Suarez, M.; Perez, G.; Johnson, E. S.; Smith, L. G. *Arch. Intern. Med.* **1985**, *145*, 2247.
19. Homeida, A. M.; El Amin, E. A.; Adam, S. E. I.; Mahmoud, M. M. *J. Comp. Pathol.* **1981**, *91*, 355.
20. Leung, D.; Hardouin, C.; Boger, D. L.; Cravatt, B. F. *Nat. Biotechnol.* **2003**, *21*, 687.
21. Bertini, V.; Buffoni, F.; Ignesti, G.; Picci, N.; Trombino, S.; Iemma, F.; Alfei, S.; Pocci, M.; Lucchesini, F.; De Munno, A. *J. Med. Chem.* **2005**, *48*, 664.
22. Buffoni, F.; Bertini, V.; Dini, G. *J. Enzyme Inhib.* **1998**, *13*, 253.
23. Bertini, V.; De Munno, A.; Lucchesini, F.; Buffoni, F.; Bertocci, B. I. P. 47906-A, 1985; E. P. 0210140, 1986; J. P. 61239891, 1986; U.S. P. 4888283, 1989. *Chem. Abstr.* **1987**, *106*, 156038m.
24. Bach, E.; Kjaer, A. *Acta Chem. Scand.* **1971**, *25*, 2629.
25. Katritzky, A. R.; He, H.-Y.; Long, Q.; Wilcox, A. L. *ARKIVOC* **2001**, *2*, 3.
26. Yoon, N. M.; Brown, H. C. *J. Am. Chem. Soc.* **1968**, *90*, 2927.
27. Lucchesini, F.; Bertini, V.; Pocci, M.; De Munno, G.; Crispini, A. *Tetrahedron* **1995**, *51*, 9757.
28. Buendia, J. *Bull. Chem. Soc. Fr.* **1966**, 3598.
29. Foster, D. J.; Reed, D. E., Jr. *J. Org. Chem.* **1961**, *26*, 252.
30. Meyers, A. I.; Himmelsbach, R. J.; Reuman, M. *J. Org. Chem.* **1983**, *48*, 4053.
31. Meyers, A. I.; Williams, B. E. *Tetrahedron Lett.* **1978**, 223.
32. Voegtle, F.; Neumann, P.; Zuber, M. *Chem. Ber.* **1972**, *105*, 2955.
33. Pocci, M.; Alfei, S.; Castellaro, S.; Lucchesini, F.; Milanese, M.; Bertini, V. *Polymer J.* **2013**, *45*, 1146.
34. Witiak, D. T.; Poochikian, G. K.; Feller, D. R.; Kenfield, N. A.; Newmann, H. A. I. *J. Med. Chem.* **1975**, *18*, 992.
35. Schow, S. R.; McMorris, T. C. *J. Org. Chem.* **1979**, *44*, 3760.
36. Dickschat, J. S.; Bode, H. B.; Kroppenstedt, R. M.; Müller, R.; Schulz, S. *Org. Biomol. Chem.* **2005**, *3*, 2824.
37. Raiford, L. C.; Clark, E. P. *J. Am. Chem. Soc.* **1923**, *45*, 1738.
38. Parsons, A. T.; Smith, A. G.; Neel, A. J.; Johnson, J. S. *J. Am. Chem. Soc.* **2010**, *132*, 9688.
39. Belke, C. J.; Su, S. C. K.; Shafer, J. A. *J. Am. Chem. Soc.* **1971**, *93*, 4552.
40. Laird, R. M.; Parker, R. E. *J. Chem. Soc.* **1965**, 4784.
41. Townsend, C. A.; Davis, S. G.; Christensen, S. B.; Link, J. C.; Lewis, C. P. *J. Am. Chem. Soc.* **1981**, *103*, 6885.
42. Hodgson, H. H.; Clay, H. *J. Chem. Soc.* **1930**, 1872.
43. Wilson, W. C.; Adams, R. *J. Am. Chem. Soc.* **1923**, *45*, 528.
44. Hodgson, H. H.; Clay, H. *J. Chem. Soc.* **1932**, 869.
45. Reinheimer, J. D.; Smith, J. C. *J. Org. Chem.* **1952**, *77*, 1505.
46. Buffoni, F.; Blaschko, H. *Proc. R. Soc. London, Ser. B* **1964**, *161*, 153.
47. Buffoni, F.; Raimondi, L. *Agents Actions* **1981**, *11*, 38.
48. Ignesti, G.; Banchelli, G.; Raimondi, L.; Pirisino, R.; Buffoni, F. *Agents Actions* **1992**, *35*, 192.
49. Pinnel, S. R.; Martin, G. R. *PNAS* **1968**, *61*, 708.
50. Melet, J.; Vianden, G. D. N. E.; Bachra, B. N. *Anal. Biochem.* **1977**, *77*, 141.
51. Pino, R.; Failli, P.; Mazzetti, L.; Buffoni, F. *Biochem. Mol. Med.* **1997**, *62*, 188.
52. Holt, A.; Sharman, D. F.; Baker, G. B.; Palcic, M. M. *Anal. Biochem.* **1997**, *244*, 384.
53. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265.
54. Lineweaver, H.; Burk, D. *J. Am. Chem. Soc.* **1934**, *56*, 658.
55. Randhawa, M. A. *J. Ayub. Med. Coll. Abbottabad* **2009**, *21*, 184.