J. Enzyme Inhibition, 1998, Vol. 13, pp. 207–216
Reprints available directly from the publisher
Photocopying permitted by license only

INHIBITORY EFFECT OF 1,3,5-TRIPHENYL-4, 5-DIHYDRO-(1H)-PYRAZOLE DERIVATIVES ON ACTIVITY OF AMINE OXIDASES

FEDELE MANNA [a,*], FRANCO CHIMENTI [b], ADRIANA BOLASCO [a], BRUNA BIZZARRI [a], OLIVIA BEFANI [c], PAOLA PIETRANGELI [c], BRUNO MONDOVI [c] and PAOLA TURINI [c]

[a] *Dipartimento di Studi di Chimica e Tecnologia delle Sostanze Biologicamente Attive, Università di Roma "La Sapienza", P.le Aldo Moro 5, 00185 Roma, Italy;*
[b] *Chimica Farmaceutica, Facoltà di Farmacia, Università di Reggio Calabria, Italy;*
[c] *Dipartimento di Scienze Biochimiche A. Rossi Fanelli, Università di Roma "La Sapienza" e Centro di Biologia Molecolare del CNR, Roma, Italy*

(Received 10 November 1997)

A new series of 1,3,5-triphenyl-4,5-dihydro-(1H)-pyrazole derivatives was synthesized to ascertain the contribution of substituted phenyl rings present on the 4,5-dihydro-(1H)-pyrazole nucleus to the monoamine oxidases inhibition and bovine serum amine oxidase inhibition. All compounds were tested on bovine brain mitochondria preparation containing flavin-monoamine oxidases and on purified bovine serum amine oxidases, taken as a model of trihydroxyphenylalanine quinone-copper-containing amine oxidases.

The 1,3,5-triphenyl-4,5-dihydro-(1H)-pyrazole derivatives showed a good inhibitory activity and belonged to the third generation of monoamine oxidase inhibitors and bovine serum amine oxidase inhibitors which have the advantage of acting through a reversible mode. Furthermore, their activity showed a good degree of selectivity towards the bovine serum amine oxidase inhibition dependent on the substituents present on the phenyl ring at position 5 of the 4,5-dihydro-(1H)-pyrazole.

Keywords: 1,3,5-triphenyl-4,5-dihydro-(1H)-pyrazole; Inhibitors; Amine oxidases

INTRODUCTION

Amine oxidases (AOs) are enzymes widely distributed among all living organisms. Their widespread occurence accounts for undoubtedly relevant

* Corresponding author. Tel.: 0039 6 49913735. Fax: 0039 6 49913763.

biological functions, such as biogenic amine metabolism. Biogenic amines are oxidatively deaminated by AOs in a reaction consuming O_2 and H_2O and producing the corresponding aldehyde, ammonia and H_2O_2.

There are two classes of amine oxidases (AOs): the flavin adenin dinucleotide-containing AOs (FAD-AOs) and the 2,4,5-trihydroxyphenylalanine quinone-copper-containing AOs (TPQ-Cu AOs). To the former sub-class belong the mitochondrial monoamine oxidases (MAO A and B) and cytosolic polyamine oxidase (PAO).[1] The development of very specific inhibitors of TPQ-Cu AOs, devoid of inhibitory effects on FAD-MAOs, may have therapeutic use as antifibrotic, antihypertensive agents and in contraception.[2,3]

It is known that some 1,3,5-trisubstituted pyrazolines show monoamine oxidase inhibitory properties that are not related to anticonvulsant activity.[4,5] In structural terms, the results of these researches indicated that the presence of an electron-donating substituent on the phenyl group at position 5 of the pyrazoline ring (see **2**) produced an increase in enzyme inhibition which was further raised by the presence of an electron-withdrawing group on the phenyl ring at position 3.[4] A subsequent search confirmed this trend for the substituents on the phenyl group at position 5 of the pyrazoline ring but showed also that the polarization of the phenyl ring at position 1, derived from the presence of an electron-withdrawing group on it, increased the inhibiting potency on MAO.[6] The good activity of the previous compounds and the absence of data on their mechanism of action, i.e. reversible or irreversible, made the synthesis of a new series of 1,3,5-triphenyl-4,5-dihydro-(1H)-pyrazoles very interesting. It could be hypothesized indeed that they might show a reversible MAO inhibition because they didn't contain any characteristic function which allowed them to give oxidized species that engage in covalent binding with the enzyme.

To ascertain the contribution of substituted phenyl rings present on the 4,5-dihydro-(1H)-pyrazole, to the inhibiting activity, new simplified structures were synthesized. Considering that the substituents on phenyl rings at position 3 and 5 of the 4,5-dihydro-(1H)-pyrazole seemed to be essential for the inhibition potency, we synthesized 1,3,5-triphenyl-4,5-dihydro-(1H)-pyrazole derivatives, characterized by the presence of a 4-chlorophenyl substituent at position 1 and of a 2-hydroxyphenyl at position 3 which seemed to be useful for the MAO inhibiting activity,[4,6] while structural modifications were introduced only on the phenyl substituent at the position 5 to study their influence on the inhibition of the enzyme.

The new 4,5-dihydro-(1H)-pyrazole derivatives were tested on bovine brain mitochondria preparation containing MAOs and on purified bovine

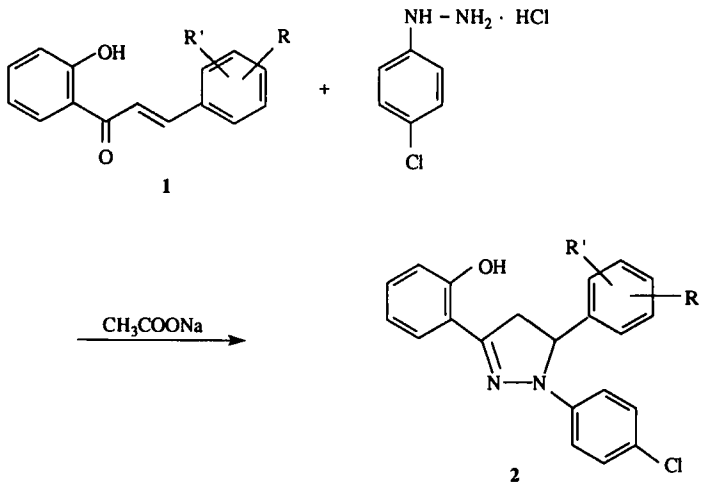
serum AO (BSAO), taken as a model of the TPQ-Cu AOs class, to study the inhibitory type, their selectivity of action between two classes of AOs and if they were characterized by a reversible activity.

MATERIALS AND METHODS

Melting points were determined in open capillary tubes with a Büchi SMP-20 apparatus and were uncorrected. IR spectra were recorded with a Perkin-Elmer 281 B instrument, ^{1}H-NMR analysis was performed on a Varian EM-390 instrument, using TMS as an internal standard. Analyses indicated by the symbols of the elements were within $\pm 0.4\%$ of the theoretical values.

1-(4-chlorophenyl)-3-(2-hydroxyphenyl)-5-(R,R′ phenyl)-4,5-dihydro-(1H)-pyrazoles (2a–u)

A mixture of 4-chlorophenylhydrazine hydrochloride (0.062 mol) in 20 ml of absolute EtOH and sodium acetate (0.062 mol) was kept at room temperature for 30 min with stirring. To a solution of the appropriate chalcone **1** (0.031 mol), obtained from condensation of *o*-hydroxyacetophenone with the required benzaldehydes, refluxed in acetic acid (45 ml), after complete dissolution of chalcone, the suspension of 4-chlorophenylhydrazine in EtOH was added dropwise. After 3 h, the solid was collected by filtration and crystallized from EtOH (Scheme 1).[7]



SCHEME 1

The physical characteristics of **2a–u** compounds are reported in Table I.

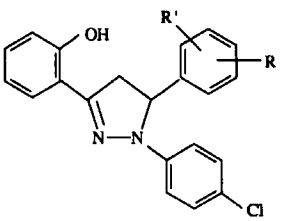
1-(4-chlorophenyl)-3-(2-hydroxyphenyl)-5-(2-chlorophenyl)-4,5-dihydro-(1H)-pyrazole (2a)

IR (KBr) cm^{-1}: 3100–3200 (OH), 1580–1600 (C=N). ^{1}H-NMR ($CDCl_3$) ppm: 3.20–3.30 (dd, 1H, H_4, $J_{H_4'H_4}$ = 18.00 Hz); 3.90–4.20 (dd, 1H, H_4', $J_{H_4'H_5}$ = 15.00 Hz); 5.20–5.50 (dd, 1H, H_5, $J_{H_4H_5}$ = 12.00 Hz); 6.80–7.50 (m, 12H, aromatic rings); 10.70 (s, 1H, OH). Anal. $C_{21}H_{16}N_2OCl_2$ (C, H, N, O).

The structures of the triaryl-4,5-dihydro-(1H)-pyrazoles **2b–u** showed similar spectroscopic data. In fact, the IR spectra showed C=N stretching bands of medium intensity at 1580–1600 cm^{-1} and strong bands in the region 2900–3100 cm^{-1} attributable to OH substituents.

In the NMR spectra the H_4'- H_4- H_5 protons showed signals as double doublets centered at ppm 3.2–3.3, 3.9–4.2 and 5.2–5.5, respectively, with

TABLE I Physical constants for 1,3,5-triphenyl-4,5′-dihydro-(1H)-pyrazole derivatives



Compound	*R*	*R′*	*Yield %*	*m.p.* °C	*Formula*
2a	H	2-Cl	39	158	$C_{21}H_{16}N_2OCl_2$
2b	H	3-Cl	51	147	$C_{21}H_{16}N_2OCl_2$
2c	H	4-Cl	86	179	$C_{21}H_{16}N_2OCl_2$
2d	H	2-Br	58	167	$C_{21}H_{16}N_2OClBr$
2e	H	3-Br	54	185	$C_{21}H_{16}N_2OClBr$
2f	H	4-Br	60	168	$C_{21}H_{16}N_2OClBr$
2g	H	2-CH_3	52	138	$C_{22}H_{19}N_2OCl$
2h	H	3-CH_3	54	131	$C_{22}H_{19}N_2OCl$
2i	H	4-CH_3	57	170	$C_{22}H_{19}N_2OCl$
2j	H	2-OCH_3	66	165	$C_{22}H_{19}N_2O_2Cl$
2k	H	3-OCH_3	50	130	$C_{22}H_{19}N_2O_2Cl$
2l	H	4-OCH_3	66	197	$C_{22}H_{19}N_2O_2Cl$
2m	H	2-NO_2	37	180	$C_{21}H_{16}N_3O_3Cl$
2n	H	3-NO_2	20	182	$C_{21}H_{16}N_3O_3Cl$
2o	H	4-NO_2	15	↑250	$C_{21}H_{16}N_3O_3Cl$
2p	H	4-$N(CH_3)_2$	73	208	$C_{23}H_{22}N_3OCl$
2q	2-Cl	4-Cl	42	166	$C_{21}H_{15}N_2OCl_3$
2r	2-OCH_3	3-OCH_3	65	152	$C_{23}H_{21}N_2O_3Cl$
2s	2-OCH_3	4-OCH_3	65	133	$C_{23}H_{21}N_2O_3Cl$
2t	2-OCH_3	5-OCH_3	58	183	$C_{23}H_{21}N_2O_3Cl$
2u	3-OCH_3	4-OCH_3	83	174	$C_{23}H_{21}N_2O_3Cl$

$J_{H'_4H_4} = 18$ Hz, $J_{H'_4H_5} = 15$ Hz and $J_{H_4H_5} = 12$ Hz. The proton of the hydroxyl group appeared as a singlet in the region ppm 10.7–10.8. The other protons were present as multiplets in the region ppm 6.8–7.5.

Preparation and Assay of Amine Oxidases

All chemicals were commercial reagents of analytical grade used without purification.

Bovine serum amine oxidase (BSAO) was purified according to Turini *et al.*,[8] whereas bovine brain mitochondria (MAO) were isolated according to Basford.[9] Activity of BSAO and MAO A and B was determined by a fluorimetric method with kynuramine as substrate.[10]

Pyrazole derivatives were dissolved in dimethyl-sulfoxide (DMSO), added to the reaction mixture, pre-incubated 10 min before adding kynuramine and then incubated for an additional 30 min for enzyme activity determination. Pargyline (1 mM) and semicarbazide (1 mM) were employed as inhibitors of respectively, mitochondrial MAO A and B, and copper-dependent AOs. The protein concentration was determined according to Goa.[11]

The inhibitory effect is expressed by I_{50}, i.e., the inhibitor concentration that reduced the enzyme activity by 50%. Dixon plots were used to estimate the inhibition constant (K_i) of the inhibitors.

RESULTS AND DISCUSSION

Three generations of MAO inhibitors (MAOIs) had been successively described. The first generation included the irreversible and non-selective inhibitors, and the second, the irreversible and selective ones. The third generation consisted of MAOIs that had the advantage of acting through a reversible and selective mode and are therefore an exciting new type of antidepressant with a safe profile compared with the previous MAOIs.[12] In particular, while the first and second generations of MAOIs engage in covalent binding to the enzyme through an intermediate reduced form of FAD,[13] the reversible and selective species of the third generation interact with FAD through weak reversible forces (electrostatic interactions and charge-transfer bonding) and form a new chemical entity, which is unstable.

1,3,5-triphenyl-4,5-dihydro-(1H)-pyrazoles **2a**–**u** showed a good inhibitory activity and belonged to the third generation of MAOIs and BSAOIs which have the advantage of acting through a reversible and selective mode.[14] In fact the results reported in Table II showed a non-competitive type of inhibition of brain MAO and BSAO activity, and the reversibility of

action was demonstrated by the fact that dialysis for 24 h in a cold room against 0.1 M potassium phosphate buffer pH 7.2 was able to restore 90–100% of the enzyme activity (data not shown). Furthermore, their activity showed a good degree of selectivity towards the BSAO inhibition, dependent on the substituents present on the phenyl ring at position 5 of the 4,5-dihydro-(1H)-pyrazole, which played a fundamental role in determining the potency and selectivity of the inhibitory activity evidenced by the IC_{50} values (Table III).

TABLE II K_i values of complexes between some 4,5-dihydro-(1H)-pyrazole derivatives and MAO or BSAO*

Compound	*MAO* K_i (M)	*BSAO* K_i (M)
2f	—	10^{-5} (±0.083)
2u	—	7.5×10^{-5} (±0.07)
2c	—	2.5×10^{-4} (±0.05)
2k	10^{-3} (±0.09)	3.5×10^{-4} (±0.05)
2m	—	$\simeq 10^{-4}$ (±0.097)
2n	7.5×10^{-5} (±0.06)	—
2r	10^{-3} (0.079)	—

* Values represent the mean of three measurements (± S.E.).

TABLE III Effect of 4,5-dihydro-(1H)-pyrazole derivatives on MAO and BSAO*

Compound	I_{50} (M)	
	MAO	*BSAO*
2a	0	5×10^{-4} (±0.5)
2b	0	10^{-4} (±0.5)
2c	0	10^{-5} (±0.1)
2d	0	10^{-4} (±0.4)
2e	$\simeq 10^{-4}$ (±0.5)	10^{-5} (±0.01)
2f	$\simeq 5 \times 10^{-4}$ (±0.12)	10^{-5} (±0.2)
2g	0	10^{-4} (±0.5)
2h	0	10^{-4} (±0.2)
2i	$\simeq 10^{-3}$ (±0.3)	5×10^{-4} (±0.6)
2j	5×10^{-4} (±0.04)	10^{-5} (±0.01)
2k	$< 10^{-3}$ (±0.2)	10^{-5} (±0.01)
2l	5×10^{-4} (±0.16)	10^{-4} (±0.5)
2m	10^{-3} (±0.1)	10^{-4} (±0.09)
2n	5×10^{-4} (±0.3)	10^{-4} (±0.01)
2o	$\simeq 10^{-4}$ (±0.25)	10^{-4} (±0.01)
2p	5×10^{-4} (±0.1)	10^{-4} (±0.2)
2q	10^{-4} (±0.035)	10^{-4} (±0.15)
2r	10^{-4} (±0.13)	10^{-5} (±0.01)
2s	0	5×10^{-5} (±0.2)
2t	0	10^{-4} (±0.01)
2u	0	10^{-5} (±0.1)

* Values represent the mean of three measurements (± S.E.).

Recent spectrophotometric and molecular orbital studies of the interaction of some reversible MAOIs with FAD showed that the initial driving forces, as the ligand approaches the receptor site, were indeed mostly electrostatic and decided the orientation of the inhibitors which successively formed a complex with FAD which was stabilized by a charge-transfer component, electrostatic interactions and van der Waals forces.[14,15]

The most probable conformation of compounds **2** is expected to be characterized by the coplanarity of the phenyl rings at positions 3 and 5 with the 4,5-dihydro-(1H)-pyrazole nucleus, stabilized by an intramolecular hydrogen bond between the hydroxyl group present on the phenyl ring at position 3 with the nitrogen atom 2 of the 4,5-dihydro-(1H)-pyrazole, while the phenyl group at position 1 should remain out of the plane. Thus, the orientation of the 1,3,5-triphenyl-4,5-dihydro-(1H)-pyrazoles **2a–u** could be given by the superimposition of the phenyl at position 5 on the attractive zones induced by the carbonyls of the flavin nucleus, while the more polarized *p*-chlorophenyl could interact with the repulsive zone which is spread over both sides of the tricyclic ring. The third phenyl ring, out of the plane, could give a hydrogen bond with other sites on FAD. The above structure and orientation are supported by a molecular modeling analysis carried out by folding and docking (Figure 1).

This interpretation could explain the increase of inhibitory activity derived from the presence of electro-donating substituents on phenyl at position 5 (**2j, 2l, 2p**), which could interact more easily with the positive attractive zone of the flavin nucleus. Moreover, this orientation didn't allow the formation of a charge-transfer bonding between the N2 of pyrazole ring and the N5 of the isoalloxazine nucleus of FAD which could be responsible for the generally low inhibitory potency of compounds **2**.

Furthermore, the lack of inhibitory activity, generally due to the presence of electron-withdrawing substituents (chloride or bromide **2a–d**) on the phenyl at position 5 of the 4,5-dihydro-(1H)-pyrazole, or to groups characterized by the absence of a disposable lone-pair (methyl **2g–2h**) which probably produced a further decrease of interactions with nitrogen atoms 1 and 3 of the flavin nucleus was in accordance with the conformation described above. But the inhibitory activity of the nitro derivatives **2m–2n–2o** and the absence of inhibition by compounds **2s–2t–2u** showed that probably other conformations and/or factors such as steric hindrance, were present which could modify the interactions of 4,5-dihydro-(1H)-pyrazole derivatives with FAD.

All pyrazole derivatives **2a–u** inhibited BSAO and the substituents present on the phenyl ring at position 5 of the pyrazoles characterized by an available

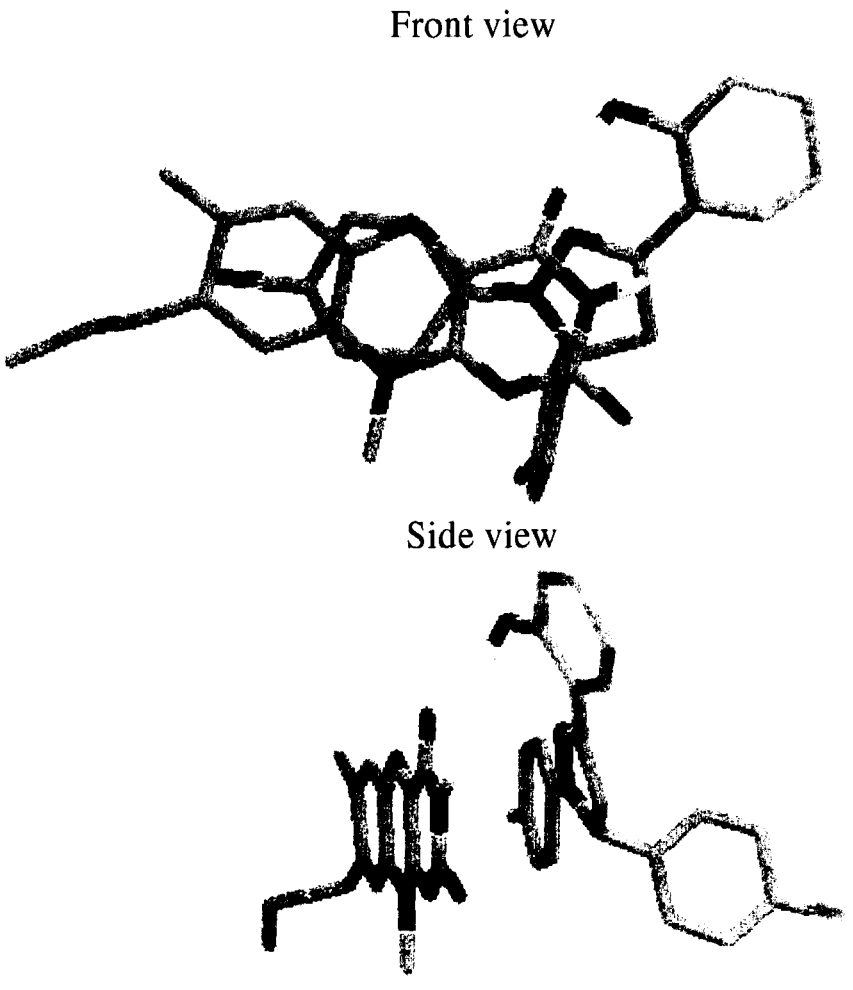


FIGURE 1 Front view and side view of compound **2c** and FAD. See Color Plate I.

lone-pair increased the inhibition ability, especially in the para position (**2c**, **2e**, **2f**, **2j**, **2k**, **2r**, **2s**, **2u**).

This behaviour suggested that the pyrazole ring interacted with one of the carbonyl group of 2,4,5-trihydroxyphenylalanine quinone (TPQ), while the lone-pair of the substituent on the phenyl group at position 5 of the pyrazole ring, formed a complex with the copper ion and consequently produced a distortion of its square planar coordination, responsible for the inactivation of BSAOs (Figure 2). Furthermore, the *p*-chlorophenyl substituent at position 1 of the pyrazole nucleus was expected to interact with the hydrophobic site of the enzyme.[17]

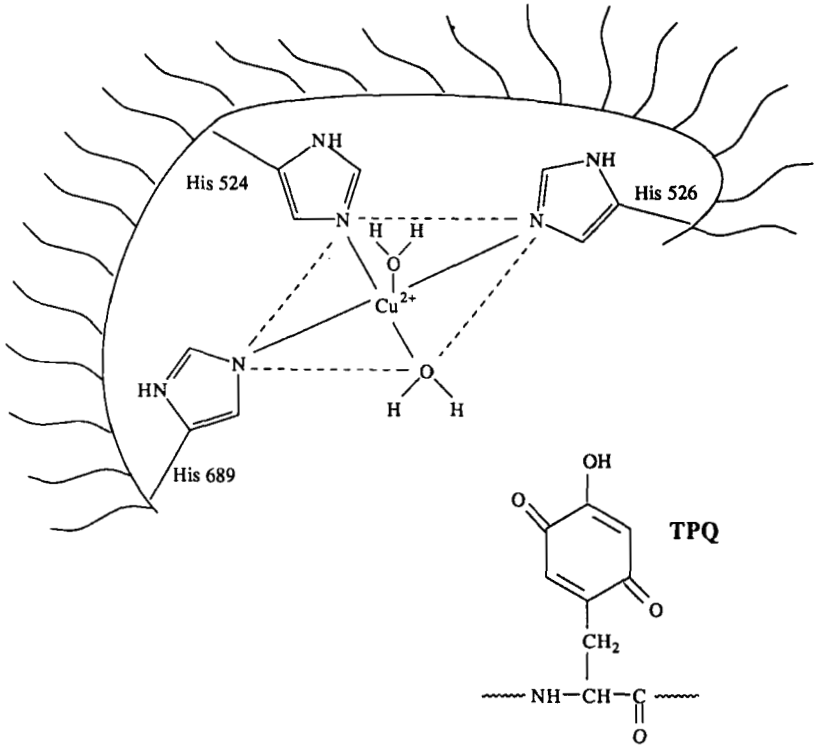


FIGURE 2 Model for the Cu(II) site in amine oxidases (from Reference 16, redrawn).

The results of this work showed that the selectivity on BSAO of 1,3,5-triphenyl-4,5-dihydro-(1H)-pyrazoles, **2**, could be obtained by structural modifications of the three phenyl substituents. Further studies aimed at clarifying the contribution of every single phenyl ring to the inhibitory activity are in progress.

Acknowledgments

This work was in part financially supported by MURST and by a CNR project on "Biotecnologia e Biologia molecolare". The skillful assistance of Mr. P. Gerosa is gratefully appreciated.

References

[1] Mondovì, B. (1985). *Structure and Function of Amine Oxidases*. CRC Press; Boca Raton, USA.

[2] Artico, M., Corelli, F., Massa, S., Stefancich, G., Avigliano, L., Befani, O., Marcozzi, G., Sabatini, S. and Mondovì, B. (1988). *J. Med. Chem.*, **31**, 802.

[3] Kavanagh, J.P., Brightwell, R., Bardsley, W.B. and Schnieden, H. (1981). *Arch. Androl.*, **7**, 51.
[4] Parmar, S.S., Pandey, B.R., Dwivedi, C. and Harbison, R.D. (1974). *J. Pharm. Sci.*, **63**, 1152.
[5] Bilgin, A.A., Palaska, E., Sunal, R. and Gumusel, B. (1994). *Pharmazie*, **49**, 67.
[6] Soni, N., Pande, K., Kalsi, R., Gupta, T.K., Parmar, S.S. and Barthwal, J.P. (1987). *Res. Commun. Chem. Pathol. Pharmacol.*, **56**(1), 129.
[7] Manna, F., Chimenti, F., Bolasco, A., Rossi, F. and Marmo, E. (1992). *Eur. J. Med. Chem.*, **27**, 633.
[8] Turini, P., Sabatini, S., Befani, O., Chimenti, F., Casanova, C., Riccio, P.L. and Mondovì, B. (1982). *Anal. Biochem.*, **125**, 294.
[9] Basford, R.E. (1967). *Meth. Enzymol.*, **10**, 96.
[10] Matsumoto, T., Suzuki, O., Furuta, T., Asai, M., Kurokawa, Y., Nimura, Y., Katsumata, Y. and Takahashi, I. (1985). *Clin. Biochem.*, **18**, 126.
[11] Goa, J. (1953). *Scand. J. Clin. Invest.*, **5**, 218.
[12] Strolin Benedetti, M. and Dstert, P.L. (1992). *Adv. Drug Res.*, **23**, 65–125.
[13] Silverman, R.B. (1988). *Mechanism-Based Enzyme Inactivation: Chemistry and Enzimology*. CRC Press; Boca Raton, USA.
[14] Moureau, F., Wouters, J., Vercauteren, D.P., Collin, S., Evrard, G., Durant, F., Ducrey, F., Koenig, J.J. and Jarreau, F.X. (1994). *Eur. J. Med. Chem.*, **29**, 269.
[15] Moureau, F., Wouters, J., Depas, M., Vercauteren, D.P., Durant, F., Ducrey, F., Koenig, J.J. and Jarreau, F.X. (1995). *Eur. J. Med. Chem.*, **30**, 823.
[16] 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. and Knowles, P.F. (1995). *Structure*, **3**, 1171–1184.
[17] Artico, M., Silvestri, R., Stefancich, G., Avigliano, L., Di Giulio, A., Maccarone, M., Agostinelli, E., Mondovi, B. and Morpurgo, L. (1992). *Eur. J. Med. Chem.*, **27**, 219–228.