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Oxidation of secondary amines by molecular oxygen and cyclohexanone monooxygenase

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Abstract—Cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* catalyzed the oxidation of tertiary and secondary amines to *N*-oxides and nitrones, respectively. The formation of a hydroxylamine intermediate was involved with secondary amines as starting substrates.

1. Introduction

The increasing demand for enantiomerically pure compounds for the pharmaceutical and agrochemical industries implies a great incentive for the study of the biotransformations. Oxidative biotransformations are amongst the most useful of all identified biologically mediated conversions.[1] They usually involve monooxygenases or dioxygenases, that catalyze the insertion of one oxygen or two oxygen atoms at a specific point of a molecule, often with high stereo and/or regioselectivity. The cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* NCIMB 9871 (CHMO) (EC 1.14.13.22) is an interesting enzyme for its application in the manufacture of fine chemicals and in organic synthesis.[2]

CHMO is a yellow FAD-containing enzyme of about 60,000 Da, it is active as a monomer and contains one tightly but non-covalently bound FAD per monomer.[2a] This enzyme catalyzes the Baeyer–Villiger oxidation of cyclohexanone with the formation of the corresponding ε-caprolactone; the only reagents consumed are O_2 and NADPH. The enzyme shows an absolute specificity for the electron donor (NADPH) and a wide specificity for the ketonic substrates.

The bacterium NCIMB 9871 can grow on cyclohexanol or cyclohexanone as sole carbon source; the key step is the ring expansion with formation of ε-caprolactone. The subsequent hydrolysis leads to 6-hydroxyhexanoate, that is oxidized to adipate, and then to the acetyl-CoA and succinyl-CoA by β-oxidation.[2] Mechanistic studies have suggested that the reactive intermediate is the FAD-4a-hydroperoxyflavin, that has a twofold activity since it can react either as a nucleophilic species, as in the case of the Baeyer–Villiger reaction and the boronic acid oxidation, or as an electrophilic species, as in the sulfoxidation reaction.[2a] More recent investigations would, however, indicate that the oxygenating intermediate is the anionic 4a-peroxide and not its protonated form, the 4a-hydroperoxyflavin.[3]

Apart from cyclohexanone, CHMO is able to transform a great variety of other substituted cyclic ketones, bicyclic ketones and aldehydes.[4] These biotransformations have wide applications for the stereoselective oxidations of prochiral ketones and constitute the only efficient methodology to resolve racemic ketones.[5] Furthermore, CHMO can oxidize a wide series of organic compounds containing electron rich heteroatoms; the sulfides are converted to sulfoxides,[2b] the sulfites to sulfates,[6] the selenides to selenoxides,[7] tertiary amines to *N*-oxides[8] and phosphines to phosphinoxides.[9] In many cases these reactions are highly enantioselective. Recently, our group has shown that the enzyme can epoxidize electron poor olefins but with severe limitations; in fact, only two compounds are accepted as substrates, namely, the dimethyl and the diethylvinylphosphonate, compounds structurally related to Fosfomycin. They are converted into the corresponding epoxides with ee >98%.[10]

The main goal of this last work was to extend the synthetic repertoire of CHMO; for this reason we have started investigating the oxidation of secondary amines to nitrones,

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a reaction previously studied with 4a-hydroperoxyflavin as oxidizing species.[11] It is known that mammalian liver contains two enzymatic systems responsible for the oxidation of *N*-substituted amine drugs, one containing a flavoprotein (NADPH cytochrome P-450 reductase) and the other a cytochrome P-450. The former catalyzes the *N*-oxidation of secondary and tertiary amines in a NADPH and O_2-dependent process similar to CHMO and the latter the *C*-oxidation of amines, that leads to *N*-dealkylation.[12]

Another point of interest is that the reaction products, the nitrones, are highly versatile synthetic intermediates, in particular as 1,3-dipoles and for the preparation of various nitrogen-containing biologically active compounds such as antibiotics, alkaloids, amino-sugars and β-lactams.[13] They have also pharmacological activity, e.g., the *tert*-butylbenzylnitrone (PBN) acts as a spin trap in physiological buffer, and exhibits antioxidant and neuroprotective activity against oxidative damage.

Nitrones are currently synthesized either by condensation of carbonyl compounds with *N*-monosubstituted hydroxylamines[14] or by oxidation of secondary amines or of the corresponding hydroxylamines.[15] The oxidative approach provides the most direct and general method for their preparation. This methodology uses hydrogen peroxide as primary oxidant in the presence of catalysts such as Na_2MoO_4 or Na_2WO_4,[15] $MeReO_3$[16] or SeO_2.[17] Very recently, alkyl hydroperoxides have been used as primary oxidant and titanium alkoxide complexes as catalyst,[18] and protected from the water formed as a co-product by the combined use of trialkanolamine ligands and molecular sieves.[18]

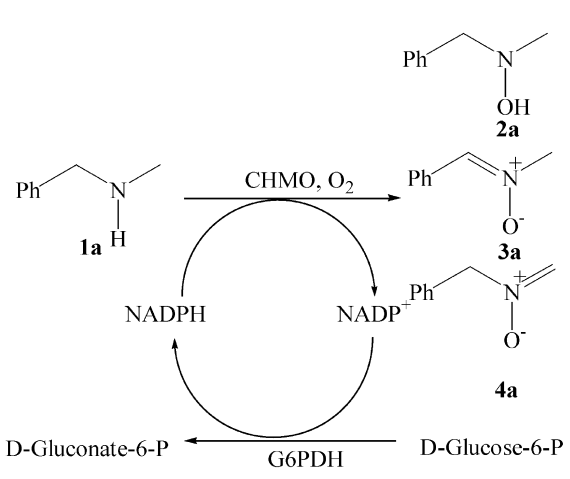
2. Results and discussion

We have examined more extensively the enzymatic oxidation of secondary amines, hydroxylamines and tertiary amines mediated by CHMO, and preliminary results have been reported.[19]

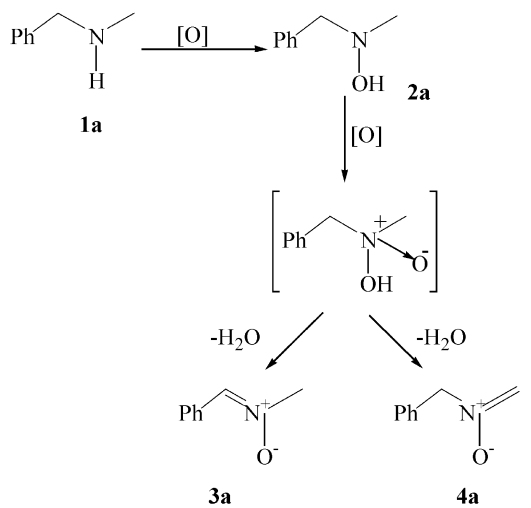
For economic reasons it is essential to regenerate NADPH in situ at the expenses of a cheap co-substrate, by means of a second enzymatic reaction This has been achieved using glucose-6-phosphate/glucose-6-phosphate dehydrogenase (G6PDH) (Scheme 1). The conversions and the identities of the products have been determined by HPLC analysis by comparison of their elution order with nitrones and hydroxylamines synthesized ad hoc. The first screening of potential amine substrates for CHMO showed that the reaction is quite sensitive to steric and electronic effects. Indeed, aliphatic amines such as diisopropylamine and pipecoline or sterically bulky amines such as dibenzylamine were not substrates for CHMO.

We have chosen *N*-methylbenzylamine (**1a**) as the model substrate in order to investigate the mechanism involved in this biooxidation. We have found that the formation of the nitrones occurs through a double oxidation, i.e., of the starting secondary amine and, then, of the hydroxylamine intermediate (**2a**) which leads to the two regioisomeric nitrones (Scheme 2). The occurrence of hydroxylamine was proved by HPLC comparison with a standard of *N*-methylbenzylhydroxylamine (**2a**). Moreover, hydroxylamine **2a** was converted by CHMO faster than the starting amine (**1a**), in agreement with its higher specificity constant value (V_{max}/K_m) (Table 1). The ratio of the two regioisomeric nitrones formed was independent of the enzymatic reaction and in favour of the most stable nitrone, in agreement with the results found by Bruice et al.[11a] in the oxidation with 4a-hydroperoxyflavin.

We have tested other substrates structurally related to the model **1a**, considering, first of all, the influence of the alkyl chain bound to the nitrogen. *N*-Ethylbenzylamine (**1b**), *N-iso*-propylbenzylamine (**1c**) and *N-n*-butylbenzylamine (**1d**) were all substrates, the specificity constant (V_{max}/K_m) order being: *N*-ethylbenzylamine>*N*-methylbenzylamine>*N-iso*-propylbenzylamine>*N-n*-butylbenzylamine (Table 1). These results were in fairly good agreement with the conversion data (Table 2). The conversions decreased when the branching of the alkyl chain was increased; *N-tert*-butylbenzylamine (**1e**) was not transformed by CHMO (Table 2). This result demonstrates the strict steric requirements of substrates. Also methyl-(1-phenyl-ethyl)-amine (**1f**) was not transformed; the substitution of a



Scheme 1. CHMO catalyzed oxidation of *N*-methylbenzylamine with in situ coenzyme regeneration.



Scheme 2. Mechanism of oxidation of *N*-methylbenzylamine by CHMO.

Table 1. Kinetic constants of CHMO with amine substrates[a]

Amines	V_{max} (μM×min^{-1})	K_m (μM)	$V_{max}/K_m \times 10^6$ (min^{-1})
N-Methylbenzylamine (**1a**)	0.21	2400	87.5
N-Methylbenzylhydroxylamine (**2a**)	0.24	2300	104.3
N-Ethylbenzylamine (**1b**)	0.20	2120	95.2
N-*iso*-Propylbenzylamine (**1c**)	0.25	4000	62.5
N-*n*-Butylbenzylamine (**1d**)	0.30	5100	58.8
N-Methyl-*p*-methoxybenzylamine (**1h**)	0.27	3170	86.4
N-Methyl-*p*-methylbenzylamine (**1i**)	0.22	2520	89.4
N-Methyl-*p*-nitrobenzylamine (**1j**)	0.21	1830	112.7
N-Methylaniline (**1l**)	0.22	2650	84.6
N,*N*-Dimethylbenzylamine (**1m**)	0.24	3060	78.4
Methyl-(4-methylthiobenzyl)-amine (**1p**)	0.40	1570	255.7

[a] For conditions see Section 3.

Table 2. CHMO catalyzed oxidation of amines

Amines	Substrate conversion (%)	Product	Product formed (%)
N-Methylbenzylamine (**1a**)	98	$C_6H_5CH_2N(OH)CH_3$ (**2a**)	80
		$C_6H_5CH{=}N(O)CH_3$ (**3a**)	10
		$C_6H_5CH_2N(O){=}CH_2$ (**4a**)	8
N-Methylbenzylhydroxylamine (**2a**)	52	$C_6H_5CH{=}N(O)CH_3$ (**3a**)	36
		$C_6H_5CH_2N(O){=}CH_2$ (**4a**)	16
N-Ethylbenzylamine (**1b**)	68	$C_6H_5CH{=}N(O)CH_2CH_3$ (**3b**)	30
		$C_6H_5CH_2N(O){=}CHCH_3$ (**4b**)	18
N-*iso*-Propylbenzylamine (**1c**)	49	$C_6H_5CH{=}N(O)CH(CH_3)_2$ (**3c**)	13
N-*n*-Butylbenzylamine (**1d**)	57	$C_6H_5CH{=}N(O)(CH_2)_3CH_3$ (**3d**)	14
		$C_6H_5CH_2N(O){=}CH(CH_2)_2CH_3$ (**4d**)	2
N-*tert*-Butylbenzylamine (**1e**)	N.R.		
Methyl-(1-phenylethyl)-amine (**1f**)	N.R		
N-Methyl-*o*-methoxybenzylamine (**1g**)	N.R		
N-Methyl-*p*-methoxybenzylamine (**1h**)	98	*p*-MeO–$C_6H_4CH{=}N(O)CH_3$ (**3h**)	10
N-Methyl-*p*-methylbenzylamine (**1i**)	68	*p*-Me–$C_6H_4CH{=}N(O)CH_3$ (**3i**)	9
N-Methyl-*p*-nitrobenzylamine (**1j**)	34	*p*-NO_2–$C_6H_4CH{=}N(O)CH_3$ (**3j**)	26
N-Methyl-*p*-chlorobenzylamine (**1k**)	N.R		
N-Methylaniline (**1l**)	60	$C_6H_5N(OH)CH_3$ (**3l**)	58
N,*N*-Dimethylbenzylamine (**1m**)	63	$C_6H_5N(O)(CH_3)_2$ (**3m**)	62
4-(Ethylaminomethyl)-pyridine (**1n**)	N.R		
Methyl-thiophen-2-ylmethylamine (**1o**)	N.R		
Methyl-(4-methylthiobenzyl)-amine (**1p**)	58	*p*-CH_3SO–$C_6H_4CH_2NCH_3$ (**5p**)	55 ee=66%

N.R.: no reaction.

benzylic hydrogen with a methyl group led to total loss of reactivity (Table 2). This fact prevented the possibility of carrying out the kinetic resolution of the starting amine.

In order to examine the stereoelectronic effects of substituents on the aromatic ring, we have investigated *N*-methyl-*o*-methoxybenzylamine (**1g**) and *N*-methyl-*p*-methoxybenzylamine (**1h**). While the *ortho*-substituted amine **1g** was recovered unchanged, the *p*-substituted one **1h** was totally transformed, giving the expected nitrone **3h** (10%) and a product deriving from the decomposition of hydroxylamine **2h** (Table 2). The instability of **2h** was demonstrated with a control experiment carried out under the same reaction conditions in the absence of CHMO. For shorter reaction times (15 min), the formation of hydroxylamine was observed together with that of its decomposition product.

The research has been extended to other substrates substituted in the para position of the aromatic ring; *N*-methyl-*p*-methylbenzylamine (**1i**) and *N*-methyl-*p*-nitrobenzylamine (**1j**) were substrates for CHMO. Again the hydroxylamine **2i** decomposed in the usual reaction conditions. In the case of *N*-methyl-*p*-chlorobenzylamine (**1k**), the starting amine was recovered unchanged. These results indicate that also the electronic requirement plays an important role. *N*-Methylaniline (**1l**) and *N*,*N*-dimethylbenzylamine (**1m**), structurally similar to the amine model **1a** were substrates for CHMO; the former reacted to give the corresponding hydroxylamine while the latter was transformed into the *N*-oxide. A further confirmation of the fact that the electronic factors are important was the total loss of reactivity in the case of 4-(ethyl-amino-methyl)-pyridine (**1n**) and of methyl-thiophen-2-yl methylamine (**1o**) (Table 2).

Finally, we took in consideration the behaviour of a substrate having two functions potentially able to undergo oxidation by CHMO, namely, two different heteroatoms, i.e. nitrogen and sulfur. Therefore, we prepared and tested methyl-(4-methylthiobenzyl)-amine (**1p**). Since the k_{cat} values of CHMO for sulfur versus nitrogen are in the 8:1 ratio,[2] a preferential attack at sulfur was expected. That was indeed the case and the corresponding sulfoxide was formed in 55% chemical yield and 66% ee (Table 2).

It should be stressed that the catalytic efficiencies of FAD-4a-hydroperoxyflavin towards secondary and tertiary amines are of the same order of magnitude (Table 1), whereas in the case of 4a-hydroperoxyflavin as oxidant, *N*,*N*-dimethyl aniline was reported to be twice as reactive with respect to *N*-methyl aniline.[12]

In conclusion, we have shown that cyclohexanone monooxygenase reacts with secondary amines, hydroxylamines and tertiary amines. The reaction has a limited versatility from the synthetic point of view since, basically, only substituted benzylic amines are accepted as substrates by the enzyme. On the other hand, we have shown that the mechanism of CHMO oxidation at nitrogen in secondary amines is similar to that of organosulfur derivatives, since both heteroatoms undergo an electrophilic attack by the terminal oxygen of the 4a-hydroperoxyflavin. In spite of the synthetic limitations, these biotransformations have a remarkable mechanistic interest if they are compared to the 4a-hydroperoxyflavin system studied by Bruice and co-workers and to hepatic flavoproteins involved in the microsomial oxidation.[12]

3. Experimental

The ^{1}H NMR spectra were recorded on a Bruker AC 300 apparatus with $CDCl_3$ as solvent. The kinetic measurements were carried out with a Jasco V-530 UV spectrophometer. Low-resolution mass spectra were run with a Fisons MD 800 spectrometer using the EI method. HPLC analyses were performed on Chiralcel OD column on a Jasco HPLC instrument (model 980-PU pump, model 975-UV detector) using *n*-hexane/2-propanol (9:1) for compounds **1a–f** and **1l–n** and *n*-hexane/2-propanol (8:2) for compounds **1g–k** and **1o–p** as the mobile phase; the flow rate was 1 ml/min and readings were made at 254 nm. Melting points were recorded on a Stuart Scientific apparatus. Chemical reactions were monitored by analytical TLC, performed on Merck silica gel 60 F_{254} plates and visualized by UV irradiation or by iodine. Columns were packed using Merck silica gel 60 (230–400 mesh) as the stationary phase and eluted using the flash chromatographic technique. CHMO was as a partially purified preparation obtained from an *E. coli* strain in which the gene of the enzyme was cloned and overexpressed.[20] Glucose-6-phosphate, $NADP^+$ and glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich. *N*-Methylbenzylamine, *N*-ethylbenzylamine, *N*-*iso*-propylbenzylamine, *N*-*tert*-butylbenzylamine, *N*-*n*-butylbenzylamine, *N*-methylaniline, 4-(ethylamino-methyl)-pyridine, *N*,*N*-dimethyl-benzylamine were purchased from Sigma-Aldrich and used without further purification.

3.1. Preparation of non-commercially available secondary amines 1g–k and 1o–p

Amines **1g–k** and **1o–p** were prepared by condensation of the corresponding aldehydes with methylamine (30% in aqueous solution), followed by reduction with $NaBH_4$ according to known methods.[21] The amines were characterized as follows.

3.1.1. *N*-Methyl-*o*-methoxybenzylamine (1g). Yellow liquid, bp 104 °C (10 Torr), (lit.[22] 103 °C). ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.41 (3H, s, NCH_3), 3.74 (2H, s, CH_2N), 3.83 (3H, s, CH_3O), 6.90 (1H, m, Ar-H), 7.23 (3H, m, Ar-H). Yield=85%.

3.1.2. *N*-Methyl-*p*-methoxybenzylamine (1h). Colourless liquid, bp 106 °C (10 Torr), (lit.[23] 105 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.68 (1H, bs, N–H), 2.44 (3H, s, NCH_3), 3.68 (2H, s, CH_2N), 6.86 (2H, d, J_{H-H}=12.0 Hz, Ar-H), 7.23 (2H, d, J_{H-H}=12.0 Hz, Ar-H). Yield=95%.

3.1.3. *N*-Methyl-*p*-methylbenzylamine (1i). Orange liquid, bp 84 °C (10 Torr), (lit.[24] 83 °C, 11 Torr). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.63 (1H, bs, N–H), 2.34 (3H, s, Ar-CH_3), 2.44 (3H, s, NCH_3), 3.71 (2H, s, CH_2), 7.18 (4H, m, Ar). Yield=95%.

3.1.4. *N*-Methyl-*p*-nitrobenzylamine (1j). Orange liquid, 147 °C (10 Torr), (lit.[25] 156–158, 15 Torr). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.43 (1H, bs, N–H), 2.46 (3H, s, NCH_3), 3.85 (2H, s, CH_2), 7.90 (2H, d, Ar-H, J_{H-H}= 9.2 Hz), 8.18 (2H, d, Ar-H, J_{H-H}=9.2 Hz). Yield=96%.

3.1.5. *N*-Methyl-*p*-chlorobenzylamine(1k). Orange liquid, bp 119 °C (10 Torr), (lit.[25] 120–123, 15 Torr). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.74 (1H, bs, N–H), 2.38 (3H, s, NCH_3), 3.66 (2H, s, CH_2), 7.22 (4H, m, Ar-H). Yield=76%.

3.1.6. Methyl-thiophen-2-ylmethylamine (1o). Yellow liquid, 79 °C (10 Torr), (lit.[26] 75–80 °C, 14 Torr). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.56 (1H, bs, N–H), 2.48 (3H, s, NCH_3), 3.95 (2H, s, CH_2), 6.96–7.23 (4H, m, Ar). Yield=99%.

3.1.7. Methyl-(4-methylthiobenzyl)-amine (1p). Yellow liquid, 108 °C (10 Torr), ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.52 (1H, bs, N–H), 2.44 (3H, s, SCH_3), 2.47 (3H, s, NCH_3), 3.70 (2H, s, CH_2), 7.24 (4H, m, Ar-H). Yield=94%.

3.2. Preparation of *N*-methyl-*N*-(1-phenylethyl)amine 1f

Amine **1l** was prepared from the corresponding primary amine by condensation with ethyl-chloroformate, followed by reduction with $LiAlH_4$.[27]

The amine was characterized as follows.

3.2.1. *N*-Methyl-*N*-(1-phenylethyl)amine 1f. Pale yellow liquid, bp 82 °C (10 Torr), (lit.[28] 83–85 °C, 15 Torr). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.35 (1H, d, CH_3, J_{H-H}= 9.9 Hz), 1.57 (1H, bs, N–H), 2.30 (3H, s, NCH_3), 3.62 (1H, q, CHN, J_{H-H}=9.9 Hz), 7.30 (5H, m, Ar). Yield=57%.

3.3. General procedure for the preparation of hydroxylamines 2a and 2h–i

$NaBH_4$ (2.0 mmol) was added to a solution of the corresponding nitrone (1 mmol) in EtOH (5 ml) and the mixture was stirred at room temperature for 5 h. The EtOH was removed under reduced pressure. H_2O was added (5 ml) and the reaction mixture extracted with CH_2Cl_2 (3×15 ml). The organic layer was dried with Na_2SO_4, filtered and the

solvent was removed under reduced pressure. Chromatography on silica gel with CH_2Cl_2/MeOH (95:5) gave hydroxylamines **2a**, **2h**, **2i**.

3.3.1. *N*-Benzyl-*N*-methylhydroxylamine 2a. Colourless solid, mp 39 °C (lit.[29] 40–41 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.58 (1H, s, NCH_3), 3.72 (1H, s, NCH_2), 7.27–7.44 (5H, m, Ar-H), OH proton signal was not observed. Yield=84%.

3.3.2. *N*-*p*-Methoxybenzyl-*N*-methylhydroxylamine 2h. White solid, mp 53–54 °C (lit.[29] 56–58 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.57 (1H, s, NCH_3), 3.67 (2H, s, NCH_2), 3.78 (3H, s, OCH_3), 7.27–7.44 (5H, m, Ar-H), OH proton signal was not observed. Yield=84%.

3.3.3. *N*-*p*-Methylbenzyl-*N*-methylhydroxylamine 2i. White solid, mp 52 °C (lit.[29] 52–54 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.32 (1H, s, Ar-CH_3), 2.57 (3H, s, NCH_3), 3.70 (2H, s, NCH_2), 7.10–7.21 (5H, m, Ar-H), OH proton signal was not observed. Yield=85%.

3.4. General procedure for the preparation of nitrones 3a–i, 3k, 3n–o, 4b, 4d and 4n

Aqueous H_2O_2 (3.06 g, 27 mmol) was added dropwise to a stirred solution of the amines **1a–i**, **1k** and **1n–o** (9.0 mmol) and $Na_2WO_4{\cdot}2H_2O$ (0.148 mg, 0.45 mmol) in MeOH (20 ml) with ice cooling The reaction mixture was stirred at room temperature for 3–12 h until TLC (CH_2Cl_2/MeOH, 95:5) revealed the complete disappearance of the amine. MeOH was removed under reduced pressure. The reaction mixture was washed with a saturated solution of Na_2SO_3 (15 ml) and extracted with CH_2Cl_2 (3×30 ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and the solvent was removed under reduced pressure. Chromatography on silica gel with CH_2Cl_2/MeOH (95:5) gave the nitrones **3a–i**, **3k**, **3n–o**, **4b**, **4d** and **4n**.

3.4.1. *N*-Benzylidenemethylamine *N*-oxide 3a. White solid, mp 82–83 °C (lit.[30] 84 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.88 (1H, s, CH_3), 7.37 (4H, m, Ar-H and CHN), 8.22 (2H, m, Ar-H). Yield=53%.

3.4.2. *N*-Benzylideneethylamine *N*-oxide 3b. Pale yellow liquid, bp 118 °C (0.8 Torr), (lit.[31] 116 °C, 0.8 Torr). ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.53 (3H, t, CH_3, J_{H-H}=7.0 Hz), 3.93 (2H, q, NCH_2, J_{H-H}=7.0 Hz) 7.33 (4H, m, Ar-H and CHN), 8.22 (2H, m, Ar-H). Yield=56%.

3.4.3. *N*-Ethylidenebenzylamine *N*-oxide 4b. Pale yellow solid, mp 76 °C (lit.[32] 77 °C). ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.95 (3H, d, CH_3, J_{H-H}=7.0 Hz), 4.89 (2H, s, NCH_2), 6.72 (2H, q, CHN, J_{H-H}=7.0 Hz), 7.37 (5H, m, Ar-H). Yield=15%.

3.4.4. *N*-Benzylideneisopropylamine *N*-oxide 3c. Yellow liquid, bp 119 °C (0.8 Torr), (lit.[31] 118 °C, 0.8 Torr). ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.50 (6H, d, CH_3, J_{H-H}=6.7 Hz), 4.17 (1H, q, NCH, J_{H-H}=6.7 Hz) 7.37 (4H, m, Ar-H and CHN), 8.24 (2H, m, Ar-H). Yield=48%.

3.4.5. *N*-Benzylidene-*n*-butylamine *N*-oxide 3d. Pale yellow solid, mp 72–73 °C (lit.[33] 73 °C). ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.94 (3H, t, CH_3, J_{H-H}=7.0 Hz), 1.44 (2H, sest., CH_2, J_{H-H}=7.0 Hz), 1.96 (2H, quint., CH_2, J_{H-H}=7.0 Hz), 3.97 (2H, t, NCH_2, J_{H-H}=7.0 Hz), 7.33 (4H, m, Ar-H and CHN), 8.24 (2H, m, Ar-H). Yield=28%.

3.4.6. *N*-Butylidenbenzylamine *N*-oxide 4d. Pale yellow solid, mp 74–75 °C (lit.[34] 76 °C). ^{1}H NMR (200 MHz, $CDCl_3$): δ 0.86 (3H, t, CH_3, J_{H-H}=7.3 Hz), 1.44 (2H, sest., CH_2, J_{H-H}=7.3 Hz), 2.37 (2H, q, CH_2, J_{H-H}=7.0 Hz), 4.80 (2H, s, Ar-CH_2), 6.63 (1H, t, CHN, J_{H-H}=7.3 Hz), 8.24 (2H, m, Ar-H). Yield=42%.

3.4.7. *N*-Benzylidene-*tert*-buthylamine *N*-oxide 3e. White solid, mp 72–74 °C (lit.[35] 75–76 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 1.61 (9H, s, CH_3,) 7.41 (3H, m, Ar-H) 7.54 (1H, s, CHN), 8.29 (2H, m, Ar-H). Yield=91%.

3.4.8. *N*-Methyl-(1-phenylethylidene) amine *N*-oxide 3f. Pale yellow solid, mp 114–115 °C (lit.[36] 115 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.43 (1H, s, CH_3), 3.63 (1H, s, NCH_3), 7.23–7.44 (5H, m, Ar-H). Yield=54%.

3.4.9. 2-Methoxybenzylidenemethylamine *N*-oxide 3g. White solid, mp 84 °C (lit.[30] 85 °C). ^{1}H NMR (200 MHz, $CDCl_3$): δ 3.77 (3H, s, NCH_3), 3.82 (3H, s, OCH_3), 6.81–6.97 (2H, m, Ar-H), 7.20 (1H, *dt*, Ar-H, J_{H-H}=9.9, 2.7 Hz) 7.78 (1H, s, CHN), 9.20 (1H, dd, Ar-H, J_{H-H}=11.9, 2.7 Hz). Yield=78%.

3.4.10. 4-Methoxybenzylidenemethylamine *N*-oxide 3h. White solid, mp 75 °C (lit.[30] 76 °C). ^{1}H NMR (200 MHz, $CDCl_3$): δ 3.81 (6H, bs, CH_3), 6.90 (2H, d, Ar-H, J_{H-H}=9.0 Hz) 7.27 (1H, s, CHN), 8.18 (2H, d, Ar-H, J_{H-H}=9.0 Hz). Yield=91%.

3.4.11. 4-Methylbenzylidenemethylamine *N*-oxide 3i. White solid, mp 117 °C (lit.[37] 119 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.42 (3H, s, Ar-CH_3), 3.83 (3H, s, NCH_3), 6.92 (2H, d, Ar-H, J_{H-H}=9.0 Hz), 7.27 (1H, s, CHN), 8.18 (2H, d, Ar-H, J_{H-H}=9.0 Hz). Yield=68%.

3.4.12. 4-Chlorobenzylidenemethylamine *N*-oxide 3k. White solid, mp 126 °C (lit.[30] 128 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 3.83 (3H, s, NCH_3), 7.37 (3H, m, Ar-H and CHN), 8.18 (2H, d, Ar-H, J_{H-H}=11.0 Hz). Yield=64%.

3.4.13. *N*-Ethyl-4-pyridylmethylidene *N*-oxide 3n. Orange liquid, bp 125 °C, 10 Torr. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.97 (3H, t, CH_3, J_{H-H}=7.3 Hz), 3.97 (2H, q, NCH_2, J_{H-H}=7.3 Hz), 7.41 (1H, s, NCH), 7.96 (2H, d, Ar-H, J_{H-H}=8.0 Hz), 8.63 (2H, d, Ar-H, J_{H-H}=8.0 Hz). EI MS: *m*/*z* (%)=150.1 (100) [M^+]. Yield=44%.

3.4.14. *N*-Ethylidenepyridyl-4-methylamine *N*-oxide 4n. Orange solid, mp 82 °C. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.96 (3H, d, CH_3, J_{H-H}=6.0 Hz), 4.83 (2H, s, NCH_2), 6.87 (1H, q, NCH, J_{H-H}=6.0 Hz), 7.25 (2H, d, Ar-H, J_{H-H}=7.5 Hz), 8.52 (2H, d, Ar-H, J_{H-H}=7.5 Hz). EI MS: *m*/*z* (%)=150.1 (100) [M^+]. Yield=22%.

3.4.15. *N*-Methyl-thienyl-2-methyleneamine *N*-oxide 3o.

Pale yellow solid, mp 118–119 °C. ^{1}H NMR (300 MHz, $CDCl_3$): 3.82 (3H, s, CH_3), 7.12 (1H, dd, Ar-H, J_{H-H}=4.8, 4.0 Hz), 7.38 (1H, d, Ar-H, J_{H-H}=4.0 Hz), 7.42 (1H, d, Ar-H, J_{H-H}=4.8 Hz), 7.83 (1H, s, CHN). EI MS: *m/z* (%)=141.1 (100) [M^+]. Yield=94%.

3.5. General procedure for the preparation of nitrones 3j and 3p

The appropriate aldehyde (1.0 mmol) was added to a solution of *N*-methylhydroxylamine hydrochloride (1.0 mmol) and sodium acetate (1.1 mmol) in EtOH (10 ml). The reaction mixture was stirred at 50–60 °C for 3 h. EtOH was removed under reduced pressure, water was added (5.0 ml) and the reaction mixture extracted with CH_2Cl_2 (3×10 ml). The organic layer was dried over anhydrous Na_2SO_4, filtered and the solvent was removed under reduced pressure. Chromatography on silica gel with CH_2Cl_2/MeOH (95:5) gave nitrones **3j** and **3p**.

3.5.1. 4-Nitrobenzylidenemethylamine *N*-oxide 3j. Yellow solid, mp 205 °C (lit.[30] 208 °C). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.42 (3H, s, Ar-CH_3), 3.83 (3H, s, NCH_3), 6.92 (2H, d, Ar-H, J_{H-H}=9.0 Hz), 7.27 (1H, s, CHN), 8.18 (2H, d, Ar-H, J_{H-H}=9.0 Hz). Yield=68%.

3.5.2. 4-Methylthiobenzylidenemethylamine *N*-oxide 3p. White solid, mp 103–104 °C (lit.[38] 105 °C). ^{1}H NMR (200 MHz, $CDCl_3$): δ 2.45 (3H, s, SCH_3), 3.80 (3H, s, NCH_3), 7.18 (2H, d, Ar-H, J_{H-H}=9.0 Hz) 7.27 (1H, s, CHN), 8.09 (2H, d, Ar-H, J_{H-H}=9.0 Hz). Yield=78%.

3.6. Preparation of (4-methanesulfinyl-benzyl)-methyl-amine 5p

Methyl-(4-methylthiobenzyl)-amine (0.420 g, 2.50 mmol) was added to a solution of $NaIO_4$ (0.560 mg, 2.60 mmol) in H_2O (25 ml) cooled with an ice bath. The reaction mixture was stirred at 0 °C for 15 h and then filtered through a Büchner funnel. The filter cake was washed with 50 ml of CH_2Cl_2. The filtrate was extracted with CH_2Cl_2 (2×25 ml), the organic layer was dried with Na_2SO_4, filtered and the solvent was removed under reduced pressure. Chromatography with CH_2Cl_2/MeOH (7:3) gave **5l** (yield=45%).

3.6.1. (4-Methanesulfinyl-benzyl)-methyl-amine 5p. Yellow liquid, bp 135 °C (10 Torr). ^{1}H NMR (300 MHz, $CDCl_3$): δ 2.45 (1H, s, NCH_3), 2.70 (3H, s, SCH_3), 3.81 (2H, s, NCH_2), 7.48 (2H, d, Ar-H, J_{H-H}=9.2 Hz), 7.59 (2H, d, Ar-H, J_{H-H}=9.2 Hz). EI MS: *m/z* (%)=183.1 (100) [M^+]. Yield=45%.

3.7. General procedure for enzymatic oxidation

The amines (15 mM) were reacted, at 25 °C, under stirring, in 0.85 ml of 0.05 M Tris–HCl buffer, pH 8.6, containing $NADP^+$ (0.5 mM), 5 units of CHMO, glucose-6-phosphate (50 mM) and 18 units of glucose-6-phosphate dehydrogenase (G6PDH) that served to regenerate the cofactor. After 24 h, the reaction mixtures were extracted with AcOEt (3×1 ml). The solvent was removed with gentle stream of N_2 and the product analyzed.

3.8. General procedure to determine the kinetic constants

The experiments were carried out in 50 mM Tris–HCl buffer at pH 8.6, 25 °C, in 1 ml cuvettes, 1 cm path length. The reaction mixture contained CHMO (30 milliunits), 0.1 mM NADPH and 0.4–5 mM substrate. The consumption of NADPH was spectrophotometrically monitored at 340 nm.

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