Laccase-Catalyzed 1,4-Dioxane-Mediated Synthesis of Belladine N-Óxides with Anti-Influenza a Virus Activity

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Abstract: Belladine N-oxides active against influenza A virus have been synthetized by a novel laccase-catalyzed 1,4-dioxane-mediated oxidation of aromatic and side-chain modified belladine derivatives. Electron paramagnetic resonance (EPR) analysis confirmed the role of 1,4-dioxane as a co-oxidant. The reaction was chemo-selective, showing a high functional-group compatibility. The novel belladine N-oxides were active against influenza A virus, involving the early stage of the virus replication life cycle.

Keywords: Oxizymes; laccase; belladine derivatives; N-oxides; influenza A virus; EPR; 1,4-dioxane

1. Introduction

Belladine 1 and norbelladine 2 (firstly extracted from the Amaryllidaceae family [1]) are bioactive precursors in the synthesis of drugs acting on the central nervous system, such as galantamine 3, lycorine 4, and haemanthamine 5 (Figure 1, Panel a) [2,3]. They are natural substances emerging in therapy, showing a cholinesterase inhibitory activity comparable to that of 3 in the treatment of Alzheimer’s disease [4,5]. In addition, a computational study suggested that quaternary belladine derivatives can interact with the neuroaminidase (NA) protein of influenza A virus, inhibiting viral release from host cell [6].

Recently, the use of N-formyl-2-bromo-O-methynorbelladine 7a in the total synthesis of 3 by a laccase (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) [7]-mediator system has been reported, focusing on the formation of the spirocyclohexadienone 6 as a tri-cyclic intermediate (Figure 1, Panel b) [8]. In this latter case, undesired side-products were produced depending on the nature of the N-substituent. Electron withdrawing group EW (R = CHO, 7a) favored the formation of phenoxy radicals and successive oxidative coupling, and the hydrolysis of the iminium ion (I) to side-chain degradation products was the only observed side-process [8,9] (Figure 1; Panel b, pathway a). Conversely, the oxidative coupling was not operative with electron-donating group ED (R = CH$_3$, 7b), in which case the isoindoline 8 was produced by an iminium-ion Polonovski transformation of the N-oxide intermediate (II) (not isolated) [10,11] (Figure 1; Panel b, pathway b).

Amine N-oxides are widely diffused in nature [12,13], and they play an important role as chiral ligands, organo-catalysts, and synths [14,15]. These compounds are synthetized using hazardous stoichiometric oxidants, or in the alternative, heavy metal catalysts and hydrogen peroxide (H$_2$O$_2$), which leads to the formation of toxic wastes and undesired by-products [16]. As an alternative, dioxygen (O$_2$) is an effective green oxidant [17–19]
when associated with laccases, favoring the formation of reactive singlet-state species in energy barrierless and green conditions. Laccases are low-cost enzymes with high catalytic activity, broad substrate specificity, and beneficial chemical and physical properties [20–29]. To the best of our knowledge, only one example of oxidation of tertiary amines by laccase has been reported; however, the yield and selectivity were low [30].

Here we describe the efficient synthesis of belladine N-oxides 9 and 12a–h (Table 2) by use of laccase from Trametes versicolor. The control of the pH of the reaction and the use of 1,4-dioxane as co-oxidant favored the stabilization of intermediate (II) by inhibition of the Polonovski transformation (Figure 1; Panel b, pathway b), affording the desired N-oxides in high yield and regio-selectivity. A modest stereo-selectivity was also observed by the use of chiral shift reagent europium tris-[3-(heptafluoropropylhydroxymethylene)-(+-)camphorate] (Eu(hfc)3) salt. Belladine N-oxides were active against Influenza A virus, and compound 12h showed the highest activity.

2. Results and Discussion

2.1. Optimization of the Reaction Conditions

The Polonovski transformation [11] of amine N-oxides occurs by two successive steps: (a) the protonation of the quaternary N-oxide moiety [31,32]; and (b) the cleavage of the iminium ion to corresponding aminium radical cation, followed by α-hydrogen elimination and skeletal rearrangement [33,34]. In order to avoid the occurrence of the Polonovski reaction in the laccase catalyzed synthesis of belladine N-oxides, the critical reaction step was expected to be the protonation of the N-oxide moiety. We started our investigation using 7b (Scheme 1) as a model substrate (general procedures are in Supplementary Materials (SM) #1, and the synthesis of 7b in SM #2).
Scheme 1. Synthesis of belladine N-oxide derivative 9 from 7b. Isoindoline 8 and 2-(2-hydroxyethoxy)acetic acid 10 were isolated as by-products.

The treatment of 7b (0.1 mmol) with laccase (1000 U·mmol⁻¹) and TEMPO (2,2,6,6-tetra methyl-1-piperidinyloxy free radical, 0.06 mmol) [35,36] at 25 °C for 3.0 h under O₂ atmosphere in 1,4-dioxane (0.5 mL) and sodium acetate buffer (2.0 mL; 0.5 M; pH 4.5) afforded 8 as the only recovered product, besides the unreacted substrate (Scheme 1; Table 1, entry 1) [8]. Examples of the retained activity of laccase in organic solvents are reported, and their advantages for the selectivity of the transformation are adequately discussed [37,38]. The oxidation with a lower amount of laccase (100 U·mmol⁻¹) in the absence of TEMPO afforded a tiny amount of N-oxide 9, alongside 8 (Scheme 1; Table 1, entry 2). Better results were obtained at pH 5.6; in this case, 9 was isolated in 28% yield (Table 1, entry 3) (NMR data of 9 are in SM #3). The reaction showed a similar behavior at higher pH. The yield of 9 was further increased by increasing the amount of 1,4-dioxane (1,4-dioxane: buffer 10:1). In this latter case, 2-(2-hydroxyethoxy)acetic acid 10 was isolated as a by-product (Scheme 1, Table 1, entry 4; NMR data of 10 are in SM #3). In addition, 9 was obtained in 56% yield using 1,4-dioxane deprived of the commercial radical scavenger butyl hydroxytoluene (BHT) (Table 1, entry 5). The use of tetrahydrofuran (THF) and acetonitrile (CH₃CN) as alternative solvents was not effective (Table 1, entry 6 and 7, respectively).

Table 1. Laccase-catalyzed 1,4-dioxane-mediated synthesis of belladine N-oxide derivative 9 starting from 7b. 

| Entry | pH  | Solvent      | Solvent/Buffer Ratio | Conversion (%) | Product(s) | Yield (%) |
|-------|-----|--------------|----------------------|----------------|------------|-----------|
| 1     | 4.5 | 1,4-dioxane  | 1:4                  | 81             | 8          | 76        |
| 2     | 4.5 | 1,4-dioxane  | 1:4                  | 45             | 8(9)       | 34(7)     |
| 3     | 5.6 | 1,4-dioxane  | 1:4                  | 50             | 8(9)       | 19(28)    |
| 4     | 5.6 | 1,4-dioxane  | 10:1                 | 60             | 8(9)|10        | 54(57)    |
| 5     | 5.6 | 1,4-dioxane  | 10:1                 | 71             | 8(9)|10        | 2(56)|8       |
| 6     | 5.6 | THF          | 10:1                 | 16             | 8(9)       | 2(56)     |
| 7     | 5.6 | CH₃CN        | 10:1                 | 5              | 8          | 2         |

1 All the reactions were performed using 0.1 mmol of the substrate in the appropriate solvent/buffer ratio (2.50 mL of total volume) and sodium-acetate buffer (0.1 M) at appropriate pH under O₂ atmosphere at 25 °C for 3 h. The experiments were conducted in triplicate. 
2 Reaction performed in the presence of 1000 U·mmol⁻¹ of laccase and TEMPO (0.06 mmol). 
3 Reaction performed in the presence of 100 U·mmol⁻¹ of laccase alone. 
4 Reaction performed in the presence of 1,4-dioxane deprived of butyl hydroxytoluene (BHT). 
5 The yield was calculated on the basis of starting mmol of the substrate.

2.2. EPR Studies

Compound 10 is reported to be the ring-opening product of 2-hydroperoxy-1,4-dioxane III (not isolated, Scheme 1) [39–41]. EPR studies with 5,5-dimethyl-1-pyrroline N-oxide (DMPO) confirmed the presence of III in the reaction mixture. As reported in Figure 2 (line a), 1,4-dioxane alone showed a tiny signal in the magnetic field range of 348–353 mT compatible with the formation of the III/DMPO-adduct. The intensity of this signal increased after the addition of laccase (line b). In addition, the same signal
was detected during the oxidation of 7b with laccase (line c). The III/DMPO-adduct was common to all cases studied with increasing intensity after the addition of laccase. From a spectroscopic point of view, the spin-trapping approach allows to trap the first radical species formed in the reaction, and the increase of intensity of the signal at 348–353 mT confirms the role of 1,4-dioxane as co-oxidant in the oxidation.

Figure 2. Line (a): CW (continuous wave) X-band (9 GHz) EPR spectrum at room temperature of 5,5-dimethyl-1-pyrroline N-oxide (DMPO) and dioxane; line (b): CW X-band (9 GHz) EPR spectrum at room temperature of DMPO, dioxane, and laccase; line (c): CW X-band (9 GHz) EPR spectrum at room temperature of DMPO, dioxane, laccase, and 7b; line (d): CW X-band (9 GHz) EPR spectrum at room temperature of DMPO, dioxane, laccase and 11h (bold line) paired to its simulation (dotted line). The spectra reported in line a and b were acquired at t = 0 min and those in line c and d at t = 180 min.

2.3. Synthesis and Characterization of Belladine N-Oxide Derivatives 12a–h

The procedure was generalized to derivatives 11a–h, covering a large panel of substituents in the aromatic ring and side chain (the synthesis of compounds 11a–h is in SM #2; NMR data of 11a–h are in SM #3). Compounds 11a–h (0.1 mmol) were treated with laccase (100 U mmol⁻¹) under O₂ atmosphere in 10:1 ratio 1,4-dioxane/sodium acetate buffer (2.50 mL; pH 5.6) at 25 °C for 3.0 h to afford N-oxides 12a–h from good to high yield (53–78%), besides to unreacted substrate (Table 2, entries 1–9) (NMR data of 12a–h are in SM #3). Isoindolines were not detected in the reaction mixture. All type of substituent patterns and side-chain length were allowed, highlighting the high chemo-selectivity and functional-group compatibility of the procedure. A further evidence of the III/DMPO-adduct is reported in Figure 2 (line d) where the EPR signal recorded during the oxidation of 11h is paired to its simulation. The intensity of the signal is higher than in the previous case. The magnetic parameters obtained from the fitting are: g = 2.0061 ± 0.0001, A_N = 1.36 mT, A_H = 1.01 mT and A_H = 0.117 mT. These parameters are typical of peroxyl radical adduct with the DMPO in organic solvents [42]. The intensity of this signal was higher than that previously observed in the oxidation of 7b, in accordance with the higher yield of 12h with respect to 9.

X-ray data confirmed the structure of 12b, which was the only product isolated as a crystal (Figure 3). The compound crystallizes in a centric space group (C2/c) containing both the enantiomers (for details of the X-ray analysis and crystallization procedure see the Section Materials and Methods).
Table 2. Synthesis of belladine N-oxide derivatives 9 and 12a–h 1.

| Entry | Compd | X | R1  | R2  | R3  | R4  | Product | Yield [%] 2 | [α]D 25 \text{°} | mmol |
|-------|-------|---|-----|-----|-----|-----|---------|-------------|--------|-------|
| 1     | 7b    | -(CH2)2- | -OH | -OCH3 | -Br | -OH | 9       | 56          | +12.8  |       |
| 2     | 11a   | -(CH2)2- | -OH | -OCH3 | -H  | -OH | 12a     | 55          | +17.4  |       |
| 3     | 11b   | -(CH2)2- | -OH | -H    | -H  | -OH | 12b     | 53          | +11.5  |       |
| 4     | 11c   | -(CH2)2- | -H  | -OH   | -H  | -OH | 12c     | 52          | +21.5  |       |
| 5     | 11d   | -(CH2)2- | -OH | -NO2  | -H  | -OH | 12d     | 70          | +6.4   |       |
| 6     | 11e   | -(CH2)2- | -H  | -H    | -H  | -OH | 12e     | 71          | +18.6  |       |
| 7     | 11f   | -CH3    | -OH | -H    | -H  | none | 12f     | 67          | -       |       |
| 8     | 11g   | -(CH2)-  | -OH | -H    | -H  | -OH | 12g     | 55          | +8.9   |       |
| 9     | 11h   | -(CH2)-  | -OH | -H    | -H  | -OH | 12h     | 78          | -7.1   |       |

1 The reactions were performed using 100 U·mmol\(^{-1}\) of laccase for 0.1 mmol of the substrate in a solvent mixture of 1,4-dioxane (2.25 mL) and sodium-acetate buffer 0.1 M pH 5.6 (0.25 mL) under O\(_2\) atmosphere at 25 °C for 3. All the reactions were conducted in triplicate. Reactions were performed in the presence of 1,4-dioxane deprived by distillation of the commercial additive butyl hydroxytoluene (BHT). 2 The yield was calculated on the basis of starting mmol of the substrate. 3 Optical rotations were recorded on a JASCO P–1000 series at 589 nm.

Figure 3. Crystal structure of 12b. Ellipsoids enclose 50% probability.

As a selected case of study, the \(^1\)H-NMR of 12b with chiral lanthanide shift reagent Eu(hfc)\(_3\) (700 µL MeOD, 13.9 mM Eu(hfc)\(_3\)) [43] showed the expected asymmetric shift pattern of the AB quartet system (4.60–4.20 ppm) for the resolution of the two enantiomers, with an enantiomeric excess (ee) of 10% (Figure 4). Polarimetric analyses of 9, 12a–e and 12g–h are reported in Table 2.

Figure 4. Panel (a): \(^1\)H-NMR spectra of compound 12b in the presence of Eu(hfc)\(_3\); panel (b): \(^1\)H-NMR spectra of compound 12b in the absence of Eu(hfc)\(_3\).
2.4. Antiviral Activity of Compound 7b and 12a–h against Influenza A Virus

Compounds 9 and 12a–h were tested against influenza A/Puerto Rico/8/34 H1N1 (PR8) virus in order to evaluate previously reported computational hypothesis about the inhibition of viral NA [6]. Influenza is responsible for large epidemics and pandemics causing severe health problems [44]. The influenza A virus (Orthomyxoviridae family) is characterized by the release of eight viral RNA segments associated with the nucleoprotein (NP) and the viral RNA-dependent RNA polymerase (RdRp) complex responsible for replication and transcription cycles [45]. Among the inhibitors of the influenza A virus, the compounds active against NA received great attention being involved in the release of viral particles from infected cells [46, 47]. In the first set of experiments, A549 cells infected with 0.001 MOI of PR8 were treated with different concentrations (range 10–40 mg/mL) of compounds 9 and 12a–h for 24 h. The expression of Hemagglutinin (HA) was analyzed by means of In Cell Western (ICW) assay (as described in the Materials and Methods section) on cell monolayers. As control of cytotoxicity, cell monolayers were also treated with the same concentrations of compounds 9 and 12a–h and stained with a Cell tag (as described in the Materials and Methods section). The supernatants of the infected A549 cells were recovered and used to newly infect a fresh monolayer of MDCK (Madin-Darby canine epithelial kidney) cells, in order to evaluate whether viral particles released from the infected cells were still infective. Table 3 shows the values of IC50, CC50, and relative SI obtained on A549 and MDCK cells. Compound 12h was the most effective against viral replication in both cell lines (IC50 range 70–73 µg/mL) with the highest SI.

Table 3. IC50, CC50 values and selective index (SI) of belladine N-oxides derivatives 9 and 12a–h

| Entry | Compd | A549 | MDCK |
|-------|-------|------|------|
|       | IC50 | CC50 | SI   | IC50 | CC50 | SI   |
| 1     | 9    | 376.88 ± 11.4 | 210.65 ± 8.1 | 0.56 | 86.84 ± 5.1 | 205.90 ± 7.9 | 2.37 |
| 2     | 12a  | 78.76 ± 4.3   | 200 ± 7.1    | 2.5  | 137.23 ± 5.9 | 137.48 ± 5.8 | 1.00 |
| 3     | 12b  | 83.23 ± 5.1   | 149.31 ± 6.2 | 1.79 | 138.28 ± 6.1 | 129.18 ± 6.0 | 0.93 |
| 4     | 12c  | 80.20 ± 4.9   | 83.79 ± 5.0  | 1.04 | 84.73 ± 4.8  | 113.86 ± 5.2 | 1.34 |
| 5     | 12d  | 1243.97 ± 30.2 | 112.69 ± 6.1 | 0.09 | 70.15 ± 4.0  | 100.51 ± 4.9 | 1.43 |
| 6     | 12e  | ND 2          | ND 2         | ND 2 | 133.32 ± 5.8 | 108.61 ± 5.1 | 0.81 |
| 7     | 12f  | ND 2          | ND 2         | ND 2 | ND 2         | ND 2         | ND 2 |
| 8     | 12g  | ND 2          | ND 2         | ND 2 | ND 2         | ND 2         | ND 2 |
| 9     | 12h  | 70.40 ± 3.1   | 287.28 ± 8.3 | 4.0  | 73.20 ± 3.3  | 220.74 ± 8.9 | 3.01 |

1 IC50: the concentration of compound causing 50% reduction of viral infection. CC50: the concentration of compound required to reduce cell viability by 50%. IC50 and CC50 are expressed in micromolar units as mean ± SD. All experiments were conducted in triplicate. The Selectivity Index (SI) of each compound was calculated as the ratio CC50/IC50. 2 ND = Not Determined.

As an example, 12h significantly reduced the HA protein expression on A549 cells (Figure 5, panel a). The released viral particles in the supernatants of A549 cells were then used to infect new monolayers of MDCK cells. After 24 h infection, the ICW assay confirmed a dose-dependent reduction of HA protein expression on MDCK cell monolayers (Figure 5, panel b), suggesting the occurrence of a block in the release of viral particles from the infected cells.

To evaluate whether the compound 12h was able to impair the cell-to-cell virus spread, higher concentrations (40 and 80 mg/mL) of 12h were added to A549 cell monolayers after viral challenge, and HA protein expression was analyzed directly on these monolayers after 24 h. The ICW assay showed a significant reduction of relative fluorescence intensity of HA protein (~50% inhibition with 80 mg/mL). Furthermore, the reduction of foci of infection in cell monolayers treated with the compound 12h compared to DMSO-treated cells [48], suggested a block in the release of viral particles probably due to an interference with the viral NA (Figure 6).
The most active derivatives against influenza A virus. The highest values of IC₅₀ and SI were lighting the specific role of the formation of gomeric products [51–53]. In addition, an appreciable stereoselectivity was observed, chemo-selectivity avoiding the undesired formation of reactive quinone species and oligo-proceeded from good to high yield, showing high functional-group compatibility and oxidation of amines [9,49,50]. Irrespective of the experimental conditions, the oxidation reaction is an alternative to the widespread reported laccase/mediator procedure for the consequence of steric hindrance of the substituents. Compounds (Figure 5, panel b), suggesting the occurrence of a block in the release of viral particles confirmed a dose-dependent reduction of HA protein expression on MDCK cell monolayers (Figure 5, panel a). The released viral particles in the supernatants of A549 cells were then fixed and stained for HA protein, as described in the Materials and Methods section. The expression of viral HA was analyzed by ICW assay, using LI-COR Image Studio Software. The percentage of relative fluorescence intensity (RFI) was calculated in comparison to untreated infected cells (considered 100%). Values are the mean ± S.D. of two replicates of one experiment of two performed (n = 2). Statistical significance of the data vs untreated infected cells was defined as * p < 0.05 and ** p < 0.001.

![Figure 5](image1.png)

**Figure 5.** The expression of Hemagglutinin (HA) by means of In Cell Western (ICW) assay in the presence of most active compound 12h; panel (A): A549 cells were infected with PR8 and treated or not with different concentrations (0–40 μg/mL) of compound 12h. After 24 infection, cells were fixed and stained for HA protein, as described in the Materials and Methods section; panel (B): Supernatants were recovered and used to infect a fresh monolayer of MDCK (Madin-Darby canine epithelial kidney) cells. The expression of viral HA was analyzed by ICW assay, using LI-COR Image Studio Software. The percentage of relative fluorescence intensity (RFI) was calculated in comparison to untreated infected cells (considered 100%). Values are the mean ± S.D. of two replicates of one experiment of two performed (n = 2). Statistical significance of the data vs untreated infected cells was defined as * p < 0.05 and ** p < 0.001.

![Figure 6](image2.png)

**Figure 6.** The expression of Hemagglutinin (HA) by means of ICW assay at late steps of viral replication. A549 cells were infected with PR8 and treated or not with 40 or 80 μg/mL of compound 12h. After 24 infection, cells were fixed and stained for HA protein, as described in the Materials and Methods section. The expression of viral HA was analyzed by ICW assay, using LI-COR Image Studio Software. The percentage of relative fluorescence intensity (RFI) was calculated in comparison to untreated infected cells (considered 100%). Values are the mean ± S.D. of two experiments, each performed in duplicate (n = 4). Statistical significance of the data vs untreated infected cells was defined as ** p < 0.001 and *** p < 0.0001.
3. Conclusions

Laccase was able to activate 1,4-dioxane as co-oxidant in the selective synthesis of belladine N-oxides 9 and 12a–h, as confirmed by the EPR detection of the corresponding DMPO/III adduct. Other organic solvents were not effective in the transformation, highlighting the specific role of the formation of III in the oxygen atom transfer process. This reaction is an alternative to the widespread reported laccase/mediator procedure for the oxidation of amines [9,49,50]. Irrespective of the experimental conditions, the oxidation proceeded from good to high yield, showing high functional-group compatibility and chemo-selectivity avoiding the undesired formation of reactive quinone species and oligomeric products [51–53]. In addition, an appreciable stereoselectivity was observed, probably due to partial inhibition of the pyramidal inversion at the nitrogen center as a consequence of steric hindrance of the substituents. Compounds 12a–c and 12h were the most active derivatives against influenza A virus. The highest values of IC₅₀ and SI were observed in the case of 12h, which is characterized by three carbon atoms in the side-chain and only one hydroxy moiety on the aromatic rings. As a general trend, the presence of at least one hydroxy moiety on the aromatic rings and two or three carbon atoms in the side-chain were required to obtain significant antiviral activity. Finally, the presence of an electron-withdrawing substituent on the aromatic ring (12d) deprived the molecule of antiviral activity.

4. Materials and Methods

4.1. Materials

Reagents and laccase from *Trametes versicolor* were obtained from commercial suppliers (Sigma-Aldrich Srl, Milan, Italy).

4.2. Enzyme Activity Assay

The enzyme activity was assayed by using 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)diammonium salt (ABTS) procedure. ABTS (5.0 mM), sodium acetate buffer (2.0 mL, pH 5.0), and the enzyme solution (200 µL) were used as a standard solution. The formation of the cation radical was detected by measuring the increase of absorbance at 420 nm (ε₄₂₀ = 36,000 M⁻¹ cm⁻¹). One unit of laccase activity has been defined as the amount of enzyme that catalyzed the oxidation of 1.0 µmol of ABTS in a 200 µL reaction mixture at 25 °C during 1.0 min.

4.3. EPR Analysis

The reaction solution was prepared adding 7b and 11h (40 mM), DMPO (60 mM), and laccase (0.12 mM) in 1,4-dioxane/sodium acetate buffer (9:1 ratio). To perform the EPR experiments, capillaries of 1.2 mm diameter were filled in and inserted in a quartz tube of 3 × 3.5 I.D. × O.D. CW (continuous wave) X-band (9 GHz). Experimental condition: 9.86 GHz 123 microwave frequency, 0.1 mT modulation amplitude, and 0.2 mW microwave power. EPR spectra were recorded at room temperature with a Bruker E580 Elexsys Series, using the Bruker ER4122 SHQE cavity. A simulation was carried out with the Easyspin simulation program 5.2.28 version, using the “garlic function”.

4.4. X-Ray Crystallography Data for Compound 12b

Compound 12b was crystallized in an NMR tube, adding 5 mg of the compound in 300µL of deuterated methanol (CD₃OD). Compound 12b was completely dissolved heating the system, and the solution was slowly cooled overnight. A single crystal of 12b was submitted to X-ray data collection on an Oxford-Diffraction Xcalibur Sapphire 3 diffractometer with a graphite monochromated Mo-Kα radiation (λ = 0.71073 Å) at 293 K. The structure was solved by direct methods implemented in the SHELXS program (Version 2013/1) [54]. The refinement was carried out by full-matrix anisotropic least-squares on F² for all reflections for non-H atoms by means of the SHELXL program [55]. The structure crystallizes in the monoclinic crystal system, space group C2/c. Crystallographic data have been
4.5. Procedure for the Synthesis of Amine N-Oxides 9 and 12a-h

Compounds 7b and 11a-h (1.0 eq., 0.1 mmol) were dissolved in a solvent mixture of 1,4-dioxane (2.25 mL) and sodium acetate buffer 0.1 M, pH = 5.6 (0.25 mL). Laccase (100 U·mmol⁻¹) was added and the mixture was gently stirred at 25 °C under O₂ atmosphere (balloon) for 3 h. After this period, the mixture was filtered and the solvent was evaporated under reduced pressure. The crude mixture was purified by silica gel column chromatography (ethyl acetate/methanol 7:1) to afford the desired products 9 and 12a-h.

Chemical reactions were monitored using thin-layer chromatography on precoated aluminum silica gel Merck 60 F254 plates and a UV lamp was used for visualization. Merck Chemical reactions were monitored using thin-layer chromatography on precoated aluminum silica gel Merck 60 F254 plates and a UV lamp was used for visualization. Merck Chemical reactions were monitored using thin-layer chromatography on precoated aluminum silica gel Merck 60 F254 plates and a UV lamp was used for visualization. Merck Chemical reactions were monitored using thin-layer chromatography on precoated aluminum silica gel Merck 60 F254 plates and a UV lamp was used for visualization. Merck Chemical reactions were monitored using thin-layer chromatography on precoated aluminum silica gel Merck 60 F254 plates and a UV lamp was used for visualization. Merck Chemical reactions were monitored using thin-layer chromatography on precoated aluminum silica gel Merck 60 F254 plates and a UV lamp was used for visualization. Merck Chemical reactions were monitored using thin-layer chromatography on precoated aluminum silica gel Merck 60 F254 plates and a UV lamp was used for visualization. Merck Chemical reactions were monitored using thin-layer chromatography on precoated aluminum silica gel Merck 60 F254 plates and a UV lamp was used for visualization. Merck
129.2 (1H, -NCH$_3$ (100 MHz, MeOD): 156.0, 132.4, 129.8, 129.6, 129.5, 128.2, 127.4, 115.2, 72.7, 69.4, 53.0, 28.0 ppm. DEPT-135-NMR (100 MHz, MeOD): 132.4 (1H, ArH, J = 12.0, 4.8 Hz), 3.13–3.08 (m, 4H, -NCH$_3$).

73.1, 69.3, 53.4, 28.1 ppm. DEPT-135-NMR (100 MHz, MeOD): 129.5 (1H, ArH, J = 12.0, 4.8 Hz), 3.13–3.08 (m, 4H, -NCH$_3$).

4.5.4. N-(4-Hydroxybenzyl)-2-(4-Hydroxyphenyl)-N-Methylethan-1-Amine Oxide (12c)

1H-NMR (400 MHz, MeOD): 7.41–7.37 (m, 2H, ArH), 7.09 (d, 2H, ArH, J = 8.4 Hz), 6.85–6.82 (m, 2H, ArH), 6.74–6.72 (m, 2H, ArH), 6.49–6.45 (m, 2H, ArH), 6.38–6.35 (m, 2H, ArH).

4.5.5. N-(3-Hydroxy-4-Nitrobenzyl)-2-(4-Hydroxyphenyl)-N-Methylethan-1-Amine Oxide (12d)

1H-NMR (400 MHz, MeOD): 8.10 (d, 1H, ArH, J = 8.8 Hz), 7.44 (d, 1H, ArH, J = 1.6 Hz), 7.24–7.22 (dd, 1H, ArH, J = 8.8, 1.6 Hz), 7.11 (d, 2H, ArH, J = 8.4 Hz), 6.75–6.72 (m, 2H, ArH).

4.5.6. N-Benzyl-2-(4-Hydroxyphenyl)-N-Methylethan-1-Amine Oxide (12e)

1H-NMR (400 MHz, MeOD): 7.61–7.59 (m, 2H, ArH), 7.49–7.42 (m, 3H, ArH), 7.10–7.07 (m, 2H, ArH), 6.75–6.71 (m, 2H, ArH), 6.51–6.47 (m, 2H, ArH).

4.5.7. 1-(3-Hydroxyphenyl)-N,N-dimethylaminoethane oxide (12f)

1H-NMR (400 MHz, MeOD): 7.28–7.24 (m, 1H, ArH), 7.02–7.00 (m, 2H, ArH), 6.91–6.88 (m, 1H, ArH), 4.35 (s, 2H, ArCH$_2$N$^-$).

4.5.8. N-(3-hydroxybenzyl)-1-(4-hydroxyphenyl)-N-methylmethylaminoethane oxide (12g)

1H-NMR (400 MHz, MeOD): 7.42–7.40 (m, 2H, ArH), 7.25 (t, 1H, ArH, J = 7.6 Hz), 6.89–6.82 (m, 5H, ArH), 4.32–4.30 (m, 4H, ArCH$_2$N$^-$).

4.5.9. N-(3-Hydroxybenzyl)-N-Methyl-3-Phenoxypropan-1-Amine Oxide (12h)

1H-NMR (400 MHz, MeOD): 7.31–7.18 (m, 6H, ArH), 7.00 (t, 1H, ArH, J = 2.0 Hz), 6.95–6.93 (m, 1H, ArH), 6.89–6.87 (m, 1H, ArH), 6.48–6.42 (m, 2H, ArH, J = 12.4 Hz).
3.32–3.20 (m, 2H, -NCH₂CH₂CH₂Ar), 3.00 (s, 3H, -CH₃), 2.69 (t, 2H, -NCH₂CH₂CH₂Ar, J = 7.6 Hz), 2.34–2.16 (m, 2H, -NCH₂CH₂CH₂Ar) ppm. ¹³C-NMR (100 MHz, MeOD): 157.3, 140.5, 130.8, 129.2, 128.2, 128.0, 125.9, 123.1, 119.1, 116.4, 72.6, 67.4, 53.2, 32.2, 24.4 ppm. DEPT-135-NMR (100 MHz, MeOD): 129.2 (-CH), 128.1 (-CH), 128.0 (-CH), 125.9 (-CH), 123.1 (-CH) 119.1, (-CH), 116.4 (-CH), 72.6 (-CH₂), 67.4 (-CH₂), 53.1 (-CH₃), 32.2 (-CH₂), 24.4 (-CH₂) ppm. Non-racemic mixture [α]²⁵D = −7.1 (c 1.00, DMSO). MS (ESI) m/z: (C₁₇H₂₁NO₂)⁻: 270.19.

4.6. Cell Cultures

A 549 (human lung epithelial carcinoma) and MDCK (Madin-Darby canine epithelial kidney) cell lines were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS); glutamine 0.3 mg/mL; penicillin 100 U/mL and streptomycin 100 mg/mL. Cell viability was estimated by trypan blue (0.02%) exclusion. All reagents were purchased from Invitrogen (Milan, Italy).

4.7. Virus Production and Infection

Influenza virus A/Puerto Rico/8/34 H1N1 (PR8 virus) was grown in the allantoic cavities of 10-day-old embryonated chicken eggs. After 48 h at 37 °C, the allantoic fluid was harvested, centrifuged at 5000 rpm for 30 min to remove cellular debris, and stored at −80 °C. Virus titration was performed by Tissue Culture Infectious Dose 50% (TCID₅₀%).

Confluent monolayers of A549 epithelial cells were challenged for 1 h at 37 °C with PR8 at a multiplicity of infection (m.o.i.) of 0.001 (TCID₅₀%/cell) incubated for 1 h at 37 °C, washed with PBS, and then incubated with medium supplemented with 2% FCS. Mock infection was performed with the same dilution of allantoic fluid from uninfected eggs. [56].

4.8. In Cell Western (ICW) Assay

The ICW assay was performed using the Odyssey Imaging System (LI-COR, Lincoln, NE, USA) as previously described [47]. Briefly, A549 or MDCK cells grown in 96-well plates (2 × 10⁴ cells/well), either infected or mock-infected (Ctr) with PR8, were fixed with 4% formaldehyde, washed, permeabilized with 0.1% Triton X-100 and incubated with PBS containing Odyssey Blocking buffer (LI-COR Biosciences, Lincoln, NE, USA). The cells were then stained at 4 °C overnight with mouse anti HA (Santa Cruz Biotechnology, Santa Cruz, CA, USA) together with Cell Tag (LI-COR Biosciences, Lincoln, NE, USA) in PBS containing 5% Odyssey Blocking Buffer. Cells were then washed and stained with a mixture of fluorochrome-conjugated secondary antibodies (fluorescence emission at 800 nm) (LI-COR Biosciences, Lincoln, NE, USA) properly diluted in Odyssey blocking buffer and fluorochrome-conjugated Cell Tag (fluorescence emission at 700 nm), for 1 h at room temperature. Cell Tag was used as control of the integrity of the cell monolayer. Subsequently, three washes with PBS plus 0.1% Tween 20 were performed and plates were analyzed by the Odyssey infrared imaging system (LI-COR). Integrated intensities of fluorescence were determined by the LI-COR Image Studio software and the relative fluorescence intensity (RFI) was expressed as a percentage compared to untreated infected cells (100%). The concentration of compounds causing a 50% reduction of viral infection (IC₅₀) and the 50% cytotoxic concentration (CC₅₀), defined as the compound concentration required to reduce cell viability by 50%, were calculated by regression analysis, considering untreated infected cells as control (100%). The Selectivity Index (SI) of each compound was calculated as the ratio CC₅₀/IC₅₀.

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