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Protective Effect of Imidazolopyrazinone Antioxidants on Ischemia/Reperfusion Injury

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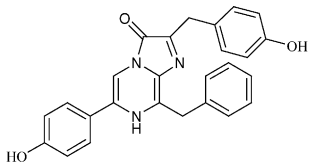
Abstract—A series of 2-substituted 3,7-dihydroimidazolo[1,2-*a*]pyrazine-3-ones has been synthesized and evaluated for their antioxidant activity. Compounds **1**–**8** are inhibitors of AAPH-induced lipid peroxidation (in vitro) and excellent protectors against microvascular damages in ischemia/reperfusion (in vivo). Hence, the bicyclic structure typical of coelenterazine (luciferin) could be considered as a useful lead in medicinal chemistry.

Oxygen-derived free radicals, usually called 'Reactive Oxygen Species' (ROS), are implicated in several pathological conditions.[1] In particular, microvascular damages due to ischemia/reperfusion have been proved to result from free radical mechanisms.[2] Therefore, radical scavengers are considered for the development of potential novel drugs in this field.[3] Both natural and synthetic antioxidants have been tested;[4] the compounds of natural origin are mostly (poly)phenolic derivatives, such as flavonoids (from plant metabolites)[5] and resveratrol (from red wine).[6] Melatonine, a tryptaminic hormone, also revealed to be efficient in preventing ischemia/reperfusion injury.[7]

Bioluminescence substrates (luciferins, lumazines)[8] are heterocyclic compounds naturally designed to react with oxygen and ROS. Accordingly, they could provide new lead-molecules in medicinal chemistry. Our interest in coelenterazine[9] (Fig. 1) analogues[10] led us to examine the protective effect of 2-substituted 3,7-dihydroimidazolo[1,2-*a*]pyrazine-3-ones on ischemia/reperfusion damage in the 'hamster cheek pouch' assay.

Imidazolopyrazinones **1**–**8** (Scheme 1) were prepared by reaction of 2-aminopyrazine with glyoxal derivatives (or the corresponding acetals) in a mixture of ethanol and aqueous HCl, at reflux. The products were precipitated by cooling at 0 °C and isolated as the hydrochloride salts.[11] They were characterized by NMR, Mass and UV spectroscopy.[12] The antioxidant character of this heterocyclic family has been previously established by a theoretical analysis of the propension of neutral compound **2** to generate diradical structures, based on the investigation of Hartree–Fock instability.[13]



Coelenterazine (natural luciferin)

Figure 1.

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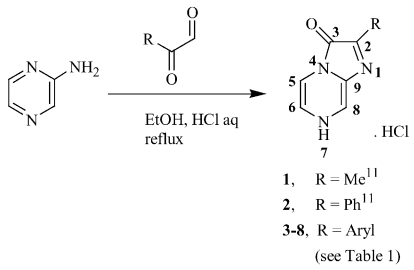


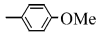
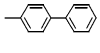
Scheme 1.

Experimentally, the antioxidant activity of imidazolopyrazinones **1**–**8** was measured by their ability to inhibit lipid peroxidation.[10b] A micellar solution of linoleic acid incubated with AAPH as free radical generator[14] produces conjugated dienes upon peroxidation; these can be monitored at 234 nm as a function of time.

Added antioxidants delayed the oxidation process;[15] the latency period associated with compounds **1**–**8** (Table 1) represents their radical scavenger ability. The lag-time induced by imidazolopyrazinones **1**, **2**, **5** and **6** could be compared to that of Trolox (water-soluble vitamin E analogue considered as a reference antioxidant). The presence of an electron-donating group (**3** and **4**) or an extra-conjugating moiety (**7** and **8**) on the aryl substituent slightly reduced the imidazolopyrazinone activity.

In vivo evaluation of compounds **1**–**8** was realized with the experimental model of 'hamster cheek pouch' allowing quantitative studies of macromolecular permeability by direct observation on microscope.[7,16] Fluorescent-labeled dextran was injected intravenously and changes in the number of microvascular leaky sites were measured after local ischemia/reperfusion. Animals were treated by gavage with the tested compounds at 3 mg/kg (or solvents), 30 min before anesthesia.

Table 1.

	R	In vitro assay[a] lag-time (min)	In vivo assay (3 mg/kg leaks inhibition (%)
1	Me	133.5 (3.3)	49[b]
2	Ph	146.2 (1.5)	79[b]; 48
3		78.7 (6.6)*	67
4		79.6 (2.8)*	85
5		110.7 (1.4)**	82
6		122.4 (8.3)**	37
7		77.5 (6.7)*	71
8		74.9 (3.9)*	57
Ref	Trolox [18]	127.5 (1.4)	—
Ref	Apocynin [19]	—	59

*Results non significantly different; ** results non significantly different.
[a]Numbers in parentheses are the standard errors of the mean calculated on triplicates.
[b]Tested at 30 mg/kg.

Results are given in percentages of inhibition of leaky sites, determined 30-min after the start of reperfusion (Table 1).[17] All compounds provided good to excellent protection against the increase in microvascular permeability due to ischemia/reperfusion. They are more or even potent compared to Apocynin (a commercially-available flavonoid usually considered as reference).[16c]

In this test (Table 1) phenyl-imidazolopyrazinone (**2**) is definitively more potent than the methyl-imidazolopyrazinone (**1**). *Para* substitution on the phenyl ring improves activity independently of the steric or electronic nature of the substituants: the methyl (**3**) and phenyl (**7**) groups are equivalent to each other and only slightly less potent than the methoxy (**4**) and chloro (**5**) groups. Only fluoro substitution (**6**) is at odd to this rule as it endows the molecule with very lower level of activity in this test, whilst this compound is one of the most potent in the reactivity assay. The lack of correlation between the in vitro and in vivo evaluation of the molecules could be explained by the nature of the tests: the AAPH test is close to a chemical measure of the antioxidant potency while the in vivo test takes into account not only the intrinsic antioxidant activity of the compound but also its oral bioavailability, metabolic stability and distribution in the specific tissue where the efficacy is measured. Compounds with equivalent intrinsic activity could manifest contrasted efficacy in this paradigm, because of differences in these other parameters. The same argument could be alluded to, regarding the activity of 2-naphthyl substituted imidazolopyrazinone (**8**) which is also less potent in vivo than compounds (**3**, **4** and **8**) presenting similar chemical reactivity.

In conclusion, imidazolopyrazinones are efficient novel antioxidants (comparable to Tolox[18]), endowed with an excellent oral bio-disponibility (similar to that of Apocynin,[19] see Table 1) in the hamster model of microvascular permeability. Other in vivo assays are in progress in view to further confirm the therapeutic potential of such heterocycles.

Acknowledgements

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12. **General procedure for the preparation of 2-aryl-3,7-dihydroimidazolo[1,2-*a*]pyrazine-3-ones.** To a solution of aminopyrazine (190 mg, 2 mmol) in ethanol (15 mL) were successively added the corresponding glyoxal derivative (3 mmol, 1.5 equiv) and HCl aq 37% (695 μL, 7.2 mmol, 3.6 equiv). The mixture was refluxed during 4 h, stirred at room temperature during the night and cooled into an ice bath during 2 h. The precipitate was filtered, washed several times with cold diethylether and dried under vacuum. The expected imidazolopyrazine salt was obtained as a colored hygroscopic solid.

Compound 3 (hydrochloride). Red solid, yield = 33%, ^{1}H NMR (CD_3OD, 500 MHz) δ 2.42 (s, 3H, CH_3), 7.35 (d, 1H, J = 8.4 Hz, H_{aryl}), 7.55 (d, 2H, J = 5.5 Hz, H_6), 8.12 (d, 1H, J = 5.5 Hz, H_5), 8.16 (d, 2H, J = 8.4 Hz, H_{aryl}), 8.64 (s, 1H, H_8), ^{13}C NMR (CD_3OD, 125 MHz) δ 21.5 (CH_3), 115.7 (C_5H), 119.7 (C_6H), 128.3 ($2C_{aryl}H$), 129.2 (C_9), 130.0 (C_8), 130.1 (C_{aryl}), 130.7 ($2C_{aryl}H$), 139.0 (C_2), 141.7 (C_{aryl}), 146.6 (C_3), MS (CI/CH_4–N_2O) m/z = 224.9 (M), 223.9 (M-1), ($C_{13}H_{11}N_3O$), UV (10^{-5} M, MeOH) λ_{max} (A) 464 (0.08), 290 (0.07), 212 (0.12).

Compound 4 (hydrochloride) Red solid, yield = 28%, ^{1}H NMR (CD_3OD, 500 MHz) δ 3,88 (s, 3H, OCH_3), 7.09 (d, 2H, J = 9.1 Hz, H_{aryl}), 7.59 (d, 1H, J = 5.6 Hz, H_6), 8.14 (d, 1H, J = 5.6 Hz, H_5), 8.25 (d, 2H, J = 9.1 Hz, H_{aryl}), 8.65 (s, 1H, H_8), ^{13}C NMR (CD_3OD, 125MHz) δ 56.0 (OCH_3), 115.6 (C_5H), 115.6 ($2C_{aryl}H$), 120.0 (C_6H), 123.7 (C_{aryl}), 129.1 (C_8H), 129.2 (C_9), 130.1 (2 $C_{aryl}H$), 137.8 (C_2), 146.0 (C_3), 163.1 (C_{aryl}), MS (APCI) m/z = 242.4 (M + 1), 241.4 (M), ($C_{13}H_{11}N_3O_2$), UV (10^{-5} M, MeOH) λ_{max} (A) 462 (0.14), 290 (0.12), 229 (0.17).

Compound 5 (hydrochloride) Red solid, yield = 49%, ^{1}H NMR (CD_3OD, 500 MHz) δ 7.56 (d, 2H, J = 8.8 Hz, H_{aryl}), 7.75 (d, 1H, J = 5.5 Hz, H_6), 8.26 (d, 2H, J = 8.8 Hz, H_{aryl}), 8.37 (d, 1H, J = 5.5 Hz, H_5), 8.97 (s, 1H, H_8), ^{13}C NMR (CD_3OD, 125 MHz) δ 117.3 (C_5H), 119.9 (C_6H), 129.8 ($2C_{aryl}H$), 130.3 ($2C_{aryl}H$), 130.6 (C_9), 130.7 (C_{aryl}), 132.8 (C_8), 137.0 (C_{aryl}), 137.4 (C_2), 144.6 (C_3), MS (APCI) m/z = 248.2 (M + 3), 246.4 (M + 1), 245.2 (M), ($C_{12}H_8ClN_3O$), UV (10^{-5} M, MeOH) λ_{max} (A) 468 (0.22), 291 (0.27), 225 (0.17).

Compound 6 (hydrochloride). Red solid, yield = 35%, ^{1}H NMR (CD_3OD, 500 MHz) δ 7.23 (t, 2H, J = 8.4 Hz, H_{aryl}), 7.32 (d, 1H, J = 5.3 Hz, H_6), 7.91 (d, 1H, J = 5.3 Hz, H_5), 8.38 (s, 1H, H_8), 8.40 (d, 2H, J = 8.4 Hz, H_{aryl}), ^{13}C NMR (CD_3OD, 125 MHz) δ 114.7 (C_5H), 116.7 ($2C_{aryl}H$, J_{CF} = 22.0 Hz), 118.1 (C_6H), 128.7 (C_8H), 129.8 (C_{aryl}, J_{CF} = 3.4 Hz), 130.3 ($2C_{aryl}H$, J_{CF} = 8.4 Hz), 130.7 (C_9), 140.2 (C_2), 149.2 (C_3), 164.8 (C_{aryl}, J_{CF} = 250 Hz), MS (APCI) m/z = 230.4 (M + 1), 229.3 (M), ($C_{12}H_8FN_3O$), UV (10^{-5} M, MeOH) λ_{max} (A) 463 (0.28), 287 (0.31), 228 (0.41).

Compound 7 (hydrochloride). Red solid, yield = 77%, ^{1}H NMR (CD_3OD, 500 MHz) δ 7.51 (d, 1H, J = 5.5 Hz, H_6), 7.55 (m, 2H, H_{aryl}), 7.88 (m, 1H, H_{aryl}), 7.95 (m, 1H, H_{aryl}), 7.97 (d, 1H, J = 8.4 Hz, H_{aryl}), 8.10 (d, 1H, J = 5.5 Hz, H_5), 8.29 (dd, 1H, J = 8.4 Hz, 1.8 Hz, H_{aryl}), 8.63 (s, 1H, H_8), 8.84 (d, 1H, J = 1.8 Hz, H_{aryl}), ^{13}C NMR (CD_3OD, 125 MHz) δ 116.0 (C_5H), 119.3 (C_6H), 125.0 (C_{aryl}), 127.9 (C_{aryl}), 128.4 (C_{aryl}), 128.4 (C_{aryl}), 128.9 (C_{aryl}), 129.5 (C_{aryl}), 129.7 (C_{aryl}), 129.8 (C_{aryl}), 130.2 (C_9), 130.4 (C_8H), 134.6 (C_{aryl}), 135.2 (C_{aryl}), 138.8 (C_2), 147.1 (C_3), MS (APCI) m/z = 262.3 (M + 1) ($C_{16}H_{11}N_3O$), UV (10^{-5} M, MeOH) λ_{max} (A) 469 (0.32), 275 (0.35), 227 (0.77).

Compound 8 (hydrochloride). Red solid, yield = 76%, ^{1}H NMR (CD_3OD, 500 MHz) δ 7.36 (d, 1H, J = 5.6 Hz, H_6), 7.37 (dd 1H, J = 7.9 Hz, 1.3 Hz, H_{aryl}), 7.47 (d, 1H, J = 7.9 Hz, H_{aryl}), 7.70 (dd, 1H, J = 7.9 Hz, 1.3 Hz, H_{aryl}), 7.78 (d, 2H, J = 8.7 Hz, H_{aryl}), 7.95 (d, 1H, J = 5.6 Hz, H_3), 8.42 (d, 1H, J = 0.8 Hz, H_8), 8.44 (d, 2H, J = 8.7 Hz, H_{aryl}), ^{13}C NMR (CD_3OD, 125 MHz) δ 114.8 (C_5H), 118.5 (C_6H), 127.9 ($2CH_{aryl}$), 128.3 ($2CH_{aryl}$), 128.7 ($2CH_{aryl}$), 128.7 (C_8H), 128.9 (C_{aryl}), 130.0 ($2CH_{aryl}$), 130.6 (C_9), 132.0 (C_{aryl}), 140.3 (C_2), 141.6 (C_{aryl}), 143.5 (C_{aryl}), 149.4 (C_3), MS (APCI) m/z = 288.3 (M + 1) ($C_{18}H_{13}N_3O$), UV (10^{-5} M, MeOH) λ_{max} (A) 475 (0.22), 308 (0.25), 240 (0.23).

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15. **Procedure for lipid peroxidation assay.** A micellar solution of linoleic acid (1.6 mM) in phosphate buffer (50 mM, pH 7.4) is incubated at 37 °C with AAPH (2 mM, 2,2′-azo-bis-(2-amidinopropane)dihydrochloride), in a microplate-based spectrophotometer (Spectra MAX 190, Molecular Devices). The peroxidation was monitored continuously at 234 nm. Antioxidant efficiency of tested compounds was evaluated by the lag phase duration until onset of linoleate peroxidation. Compounds were all tested at a final concentration of 5 μM. They were first dissolved in ethanol (final concd 0.05%), then in the phosphate buffer. Controls contained the same ethanol concentration. In parallel to the measurement at 234 nm (production of dienes), the disappearance of imidazolo-pyrazinones was monitored at 420 nm. ANOVA and Tukey tests were used for comparing the activity of compounds among groups.

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17. **Permeability in the hamster cheek pouch submitted to ischemia/reperfusion (I/R).** Male Syrian hamsters (Mesocricetus auratus, Engle Labs Farmersburg, Indianapolis, USA), 7–10 weeks old, weighing 90–130 g, were used for these studies. Anesthesia was induced by an intraperitoneal injection of 0.1–0.2 mL of sodium pentobarbital (Sanofi, France, 60 mg/mL)

and maintained with α-chloralose (100 mg/kg) administered through the femoral vein. The femoral artery was cannulated for measurement of arterial blood pressure. Throughout surgery and the subsequent experiment, the temperature of the animals was kept at 37.5 °C with a heating pad controlled by a rectal thermistor. A tracheal tube was inserted to facilitate spontaneous breathing.

The hamster was placed on a microscope stage. The cheek pouch was gently averted and pinned with 4–5 needles into a circular well filled with silicone rubber to give a flat bottom layer thus avoiding stretching of the tissue but preventing shrinkage. In this position, the pouch was submerged in a superfusion solution that continuously flushed the pool of the microscope stage.

The superfusion solution was a *N*-2-hydroxy-ethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES)-supported HCO_3^--buffered saline solution (composition in mM/NaCl 110.0, Kcl 4.7, $CaCl_2$ 2.0, $MgSO_4$ 1.2, $NaHCO_3$ 18.0, Hepes 15.39 and Hepes Na^+-salt 14.61) whose temperature was maintained at 36.5 °C and the superfusion rate was 6 mL/min. The pH was set to 7.40 by bubbling the solutions continuously with 5% CO_2 in 95% N_2.

Thirty min after the completion of the preparative procedure, fluorescein isothiocyanate (FITC)-dextran, molecular weight 150 000 dalton (Sigma), with a degree of substitution of 2 FITC molecules per 1000 glucose molecules in the polysaccharide chain was given in a dose of 25 mg/100 g body weight as an intravenous injection of 5% solution in 0.9% saline.

Observations were made with a Leitz Ortholux microscope with ×3.5 objective and ×10 oculars. The light source was a 100 W mercury DC lamp (Irem model EL-XH5 P/L). The specific light filters used for observations in fluorescent light (Leitz BG12, BG38, GG455 and KP490) were positioned between the light source and the condensor to give a light for optimal excitation at 490 nm of FITC-dextran. A barrier filter (K530) was placed between the objective and the eyepieces.

Observations of the number of leakage sites (=leaks) were made by scanning manually the total observation area twice at ×35 magnification. The fluorescent spots formed at leakage sites could be traced when they reached a certain minimal size and fluorescent intensity. Each site was classified as a leakage site when its diameter was larger than 100 μm. The number of leakage sites is reported per square centimeter. All hamsters with the prepared area showing spontaneous nonfading leaks or more than 10 fading leaks during the first 30-min control period, after FITC-dextran was given, were discarded.

A group of three animals were treated by gavage with 0.2 mL of a solution containing the compound at 3 mg/kg while another group of three animals received 0.2 mL of the solvent. Each animal was treated with the compound or the solvent 30 min before anesthesia. The preparations were used to investigate the effect of local ischemia, which was obtained by a cuff, made of thin latex tubing, mounted around the neck of the everted pouch where it leaves the mouth of the hamster. The cuff can be placed without any visible interference with local blood flow. The intratubular pressure of the cuff can be rapidly increased by air compression using a syringe, and also be rapidly decreased at evacuation; an intratubular of 200–220 mmHg resulted in a complete arrest of the microvascular flow within a few seconds. Throughout the 30-min occlusion period, minor adjustments of blood movement could be observed in the larger blood vessels. At evacuation, an immediate increase in blood flow occurred and the blood flow eventually returned to the pre-occlusion values. The maximal number of leaky sites per cm^2 was determined 30 min after the start of reperfusion. The mean of the leaky sites was calculated for the solvent and the treated groups of animals and the percentage of inhibition was calculated as follows:

[1−(mean treated)/(mean control)]×100.

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