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Coelenterazine (Marine Bioluminescent Substrate): A Source of Inspiration for the Discovery of Novel Antioxidants

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ABSTRACT Coelenterazine and derivatives were initially considered in the scientific community for their (bio)luminescent properties. Now, another interest of such hetero-bicycles has been pointed out by the discovery of remarkable antioxidative properties, and an unique mode of action as a "cascade": the mother-compound (imidazolopyrazinone) is transformed by ROS into a daughter-compound (2-amino-pyrazine) also endowed with antioxidative properties. This review illustrates the therapeutic potential of synthetic imidazolopyrazinones (coelenterazine analogues): chemical reactivity assays with singulet oxygen, radical anion superoxide, peroxynitrite, and radicals formed during lipid and LDL peroxidation, cellular tests of protection against oxidative stress using keratinocyte, hepatocyte, neuronal and erythrocyte cells, and finally in vivo evaluation in a hamster model of ischemia-reperfusion, are fully described.

KEYWORDS Coelenterazine, Coelenteramine, Imidazolopyrazinone, Amino-pyrazine, Antioxidant, Cell protection against oxidative stress, In vivo protection against ischemia-reperfusion

INTRODUCTION

Reactive oxygen species (ROS) generated in biological systems during normal oxygen metabolism have been implicated in aging and degenerative diseases. Indeed, free radical reactions, especially with participation of ROS, cause damages to lipids, proteins, membranes and nucleic acids (Gutteridge, 1999; Halliwel & Gutteridge, 1999; Kehrer, 1993; Scott, 1997). Natural protective systems towards ROS exist (Cadenas, 1995; Rice-Evans & Burdon, 1994), with radical scavengers such as vitamin E (α-tocopherol) and vitamin C (ascorbic acid), as well as with enzymatic controls of the cellular oxidative level and enzymatic repairing processes. But, under pathological conditions, ROS can be produced in excessive concentrations (or in wrong locations), causing the annihilation of the natural endogeneous defences. The resulting oxidative stress is directly or indirectly associated with the pathogenesis of various disorders (Davies, 1995; Sies, 1991): cardiovascular diseases (Cantor et al.,

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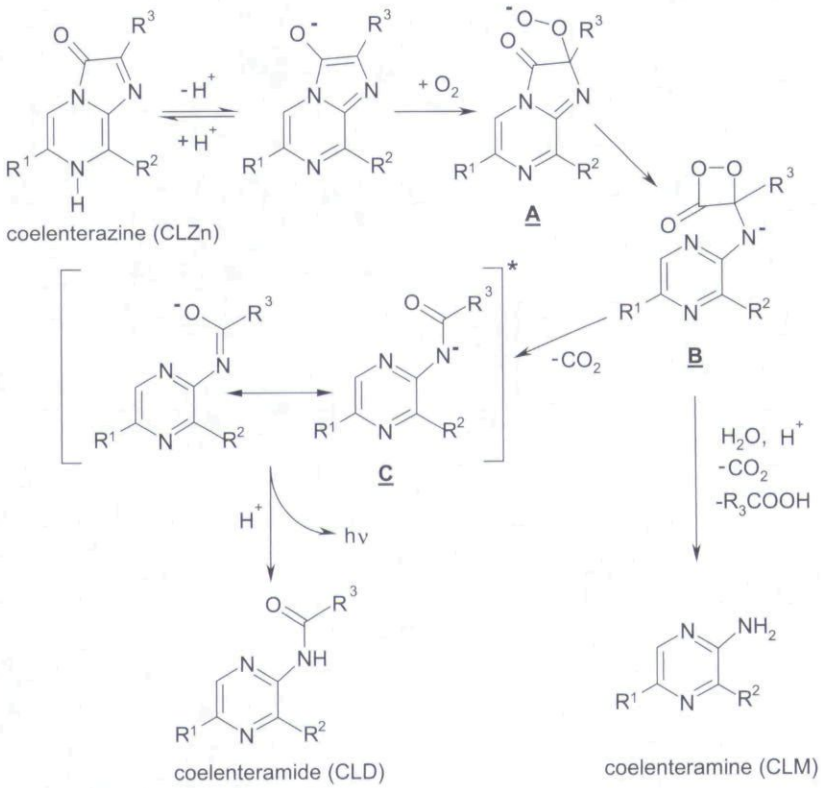
2003), reperfusion injuries, Alzheimer's, and other neuro-degenerative diseases (Metodiewa & Koska, 2000), cancers and inflammatory diseases (Floyd & Hensley, 2002; Golden et al., 2002; Poulsen et al., 1998). Accordingly, antioxidant-based treatments are regarded as possible therapies. Also, the protective role of antioxidants is considered with a growing interest for prevention purposes (Frei, 1994; Gordon, 1996).

Naturally-occurring antioxidants (Bors et al., 1996), i.e., α-tocopherol (Bowry & Ingold, 1999), ascorbic acid, flavonoids (Milane et al., 2004), carotenoids, phenols (resveratrol from wine), polyphenols (catechins from green tea), and glutathione have been studied, as well as related synthetic derivatives designed to optimize the antioxidant activity and the bioavailability properties (Hussain et al., 2003; Thomas, 1997). Generally, these antioxidants come from dietary sources (Gordon, 1996).

Since ever, the main source of naturally-occurring active molecules has been indeed our terrestrial environment of plants and animals. The systematic investigation of the marine world for the discovery of novel biologically active compounds has emerged more recently, about thirty years ago (Newman et al., 2000). In the course of our studies devoted to marine bioluminescent organisms, we investigated the properties of coelenterazine (De Wergifosse et al., 1999), the luminescent substrate of luciferases, and proposed an evolutionary model: the primary function of coelenterazine was that of an antioxidant, protecting tissues of animals thriving in the surface waters of the oceans against the acute problems of oxygen toxicity occurring in this environment. Its present day bioluminescent role could have been later acquired when the organisms colonized the deep-sea areas where both the oxidative dangers and the solar irradiance decrease. In these conditions, a selective advantage could be conferred to species able to catalyze the light-emitting oxidation of coelenterazine. Our evolutionary model was supported by the demonstration that coelenterazine is endowed with remarkable antioxidative properties (De Wergifosse et al., 1999).

Bioluminescence (Campbell et al., 1994), this beautiful natural phenomenon of light emission from living organisms for communication, reproduction, or protection purposes, is thus the starting point of our thinking in medicinal chemistry in view to propose



SCHEME 1 The Chemiluminescent Reaction of Coelenterazine *1*. R^1=Ph*p*OH; R^2=CH_2Ph; R^3=CH_2Ph*p*OH.

novel lead compounds in the field of therapeutic antioxidants. The general mechanism of bioluminescence has been well established, and is similar for all insects (Orlova et al., 2003) and marine organisms (Vysotski & Lee, 2004): light arises from the oxidation of a substrate (called luciferin) catalyzed by an enzyme (called luciferase) that results in the formation of a product in an electronically excited state. Light is emitted when this excited state decays to the ground state. Since luciferins are naturally designed to react with oxygen, they are intrinsically potential antioxidants. We decided to explore this idea with coelenterazine (CLZn) as the natural model (Scheme 1).

COELENTERAZINE, BIOLUMINESCENCE, AND OXIDATIVE DEGRADATION

Coelenterazine ***1***, i.e., 3,7-dihydro-2-(*p*-hydroxybenzyl)-6-(*p*-hydroxyphenyl)-8-benzylimidazolo-[1,2-a]pyrazin-3-one, is well known for its role as luciferin among the marine bioluminescent, mainly deep-sea, animals (Rees & Thompson, 1994; Wilson & Hastings, 1998). Coelenterazine was first isolated from the jellyfish *Aequora* where it constitutes the chromophore of the photoprotein aequorin, a calcium-binding enzyme. Its central core consists of an imidazolopyrazinone (IMPZ) nucleus to which are connected three lateral groups R^1, R^2, and R^3 (Scheme 1). It is found in a wide range of phyla, such as fishes, protozoans, coelenterates, molluscs, and chaetognats (Campbell & Herring, 1990; Haddock & Case, 1994; Rees et al., 1990, 1992; Shimomura et al., 1980). The occurrence of ***1*** in phylogenetically-distant animals, as well as in non-bioluminescent organisms (Shimomura, 1987) may, in many cases, be the result of dietary transfer through the marine food chain (Thompson & Rees, 1994). Whereas tissue distribution studies have shown that coelenterazine ***1*** is abundant in the light organs of bioluminescent animals, the main storage organ of the luciferin is the liver (or digestive gland) in fish, shrimps, and cephalopods (Thomson et al., 1997). In these organs, ***1*** is found mainly as conjugated derivatives, which are far more resistant to autoxidation than the free form (Rees & Thompson, 1994). The widespread distribution of ***1*** in marine animals, as well as the existence of stabilizing mechanisms of this luciferin in the liver of non-bioluminescent organisms, suggested the involvement of this molecule in other, non-luminescent functions (Janssens et al., 2000; Rees et al., 1998).

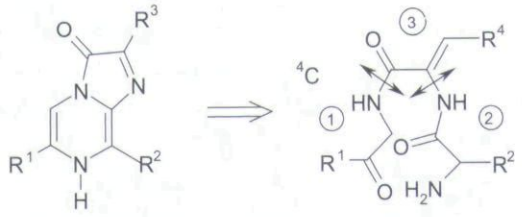
At the present time, chemi- and bioluminescence methods are widely used in biomedical sciences, for both routine analyses and research applications (Kricka, 2000; Trowern, 2002). Coelenterazine ***1*** and recombinant aequorin were involved in assays for monitoring calcium concentration in mammalian cells or subcellular compartments (Robert et al., 2000; Stables et al., 2000). Indeed, the cloning of the aequorin gene in 1985 opened the way of the stable expression of this photoprotein in cell lines and even entire organisms (Dupriez et al., 2002). Using mitochondrially expressed aequorin and a CLZn derivative, functional assays have been validated for the screening of G-protein-coupled receptors, ion channels, and tyrosine kinase receptors, as well as for their pharmacological characterization in agonists and antagonists detection assays (Dupriez et al., 2002).

Fluorescence resonance energy transfer (FRET) techniques based on bioluminescence have been also developed for assaying protein–protein interactions in living cells and in real time; such methods make use of a luciferase (genetically fused to a protein of interest) and its natural substrate CLZn (Xu et al., 2003).

Recent studies about the mechanism of CLZn transport and cell permeation reported that living cells stably transfected wih a codon-humanized *Rluc* (*Renilla* luciferase) show CLZn-mediated bioluminescence in a highly *MDR1* Pgp (multidrug resistance 1 P-glycoprotein)-modulated manner. Therefore, using CLZn and noninvasive bioluminescence imaging in vivo, it was possible to directly monitor tumor-specific Pgp transport inhibition in living mice (Pichler et al., 2004).

The previous examples illustrate well the growing interest of CLZn in various bio-assays useful in modern medicinal chemistry. The mechanism of light-emission from CLZn has been the subject of a tremendous amount of chemical works, using mainly mass and NMR spectroscopic analyses (Hart et al., 1978; Hirano et al., 1993; Hori et al., 1973; Isobe et al., 1998; Ohmiya & Hirano, 1996; Teranishi et al., 1994; Usami & Isobe, 1996).

As shown in Scheme 1, the luminescence of CLZn ***1*** can be triggered by oxygen in the absence of



SCHEME 2 **Biomimetic and Modular Synthetic Strategy Towards Imidazolopyrazinones (IMPZs)**

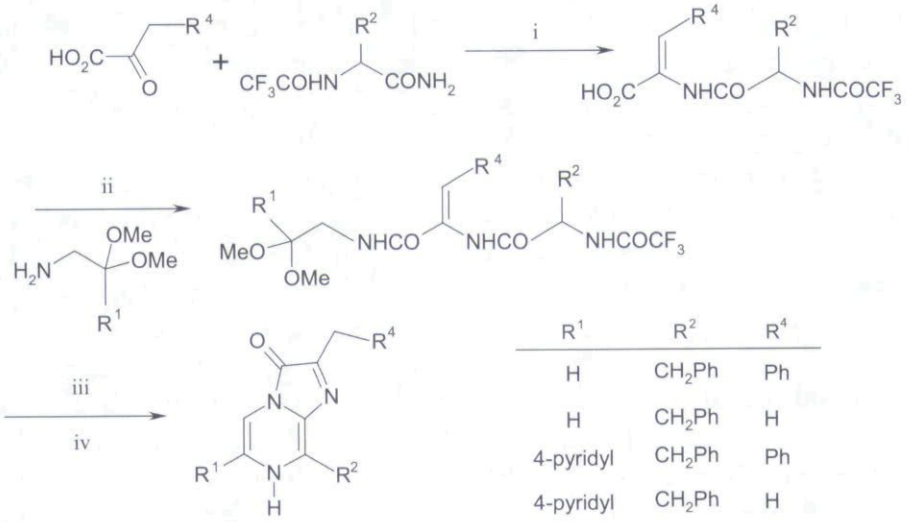
luciferase enzyme, in an aprotic polar solvent. The first step of the process is the formation of a peroxide ***A*** by reaction of the anion of ***1*** with oxygen; the second step is the conversion of the peroxide into a dioxetanone ***B*** which rapidly collapses into carbon dioxide and excited coelenteramide anion ***C***. This species is the light emitter. Thus the main products of the oxidative degradation of CLZn ***1*** are CO_2 and coelenteramide (CLD), after decay and reprotonation. The formation of coelenteramine (CLM) as by-product has been mentioned occasionally (Usami & Isobe, 1996).

We have observed that CLZn also luminesces in the presence of ROS in aqueous solution (De Wergifosse et al., 1999; Ghislain et al., 1995; Rees et al., 1998) and behaves as an excellent scavenger towards lipid-derived radicals formed during lipid peroxidation (De Wergifosse et al., 2004). We have followed by high pressure liquid chromatography (HPLC) the products formed during the AAPH-induced oxidation of a lineolate emulsion in the presence of CLZn analogues [R^1=Ph*p*OH; R^2=CH_2Ph; R^3=Ph (De Wergifosse et al., 2004), and R^1=Ph*p*OH; R^2=H; R^3=Ph (Moens et al., 2001)]. Their consumption was accompanied by the concomitant formation of CLM (or analogue; R^2=H), besides the expected CLD derivatives (R^3=Ph). The formation of benzoic acid (R^3-CO_2H; R^3=Ph) in an equivalent amount as CLM (or analogue; R^2=H) was also detected (Moens et al., 2001). In our experimental conditions, CLD was not hydrolyzed into CLM. We have thus pointed out a parallel route of CLZn oxidative degradation in aqueous media, leading to CLM most probably via reprotonation and hydrolysis of the dioxetanone intermediate ***B*** (De Wergifosse et al., 2004). Since the in situ produced CLM was also endowed with chain-breaking properties, it prolonged the antioxidant activity of the mother compound CLZn (De Wergifosse et al., 2004; Dubuisson et al., 2004). Coelenteramide was devoid of antioxidant properties. In fact, we further demonstrated that aminopyrazine derivatives related to CLM constitute a novel class of phenolic antioxidants which can be exploited as such (Dubuisson et al., 2004). This crucial discovery raised our interest in the study of CLZn ***1*** and related IMPZs as an original system of antioxidants acting in cascade (see p. 839).

The development of IMPZs in this context required first the setting up of a practical synthesis of compounds available for testing.

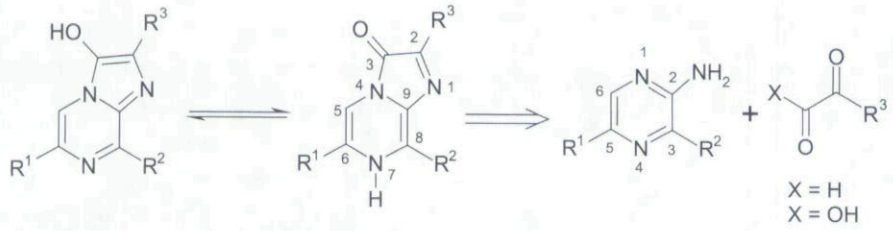
SYNTHESIS OF IMPZS

A biomimetric and modular approach towards IMPZs, based on the double intramolecular



R^1	R^2	R^4
H	CH_2Ph	Ph
H	CH_2Ph	H
4-pyridyl	CH_2Ph	Ph
4-pyridyl	CH_2Ph	H

SCHEME 3 **Synthesis of IMPZs. Reagents and Conditions: (i) *p*TosOH Catal., Benzene, Reflux; (ii) WSC, HOBT, Et_3N, THF, 20°C; (iii) TMSI, $CHCl_3$, 50°C; (iv) K_2CO_3, CH_3CN-H_2O (10:1), 80°C then HCl-H_2O Work Up.**

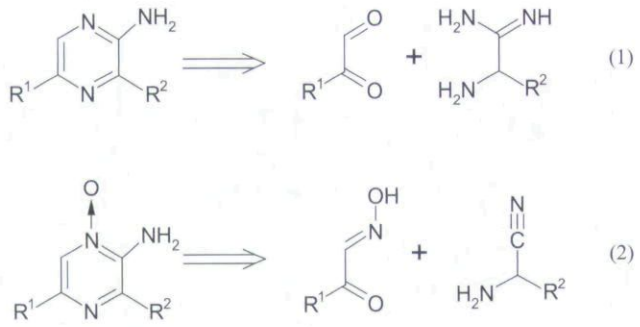


SCHEME 4 General Synthetic Strategy Towards IMPZs.

dehydration of pseudo-dehydrotripeptide precursors, was an attractive strategy to be explored with the view of a possible development in parallel, automated, synthesis (Scheme 2). On the basis of the pioneering work of McCapra (McCapra & Manning, 1973; McCapra & Roth, 1972) and the recent publications on the biosynthesis of green fluorescent protein (GFP) chromophore (Donnelly et al., 2001; El Yazal et al., 2000), we identified three building blocks susceptible to be linked by using the peptide synthesis methods, namely a β-aminoketone unit (bearing the R^1 substituent), an α-aminoamide unit (bearing the R^2 substituent), and a pyruvic unit (bearing the R^3 substituent precursor; $R^3{=}CH_2R^4$). Condensation of the pyruvic acid derivative with the N-protected α-aminoamide gave a dehydrodipeptide (Scheme 3; step 1) which was further coupled to the β-amino-ketone (or aldehyde) derivative protected as dimethylacetal (step 2). Carbonyl unmasking by treatment with trimethylsilyl iodide (step 3), followed by trifluoroacetamide deprotection by basic hydrolysis (step 4), furnished the cyclization precursor (shown in Scheme 2); this

TABLE 1 Synthetic IMPZ Derivatives

Compound	R^1	R^2	R^3	References
1 (CLZn)	Ph*p*OH	CH_2Ph	CH_2Ph*p*OH	(Adamczyk et al., 2003; Gonzalez-Trueba et al., 1996; Inoue et al., 1975)
2	H	H	CH_3	(Devillers et al., 1999, 2001)
3	H	H	Ph	(Devillers et al., 1999, 2001)
4	Ph*p*OH	CH_2Ph	CH_3	(De Wergifosse et al., 2004; Inoue et al., 1975)
5	Ph*p*OH	CH_2Ph	Ph	(De Wergifosse et al., 2004; Inoue et al., 1975)
6	Ph*p*OCH_3	CH_2Ph	CH_3	(De Wergifosse et al., 2004; Inoue et al., 1975)
7	Ph*p*OCH_3	CH_2Ph	Ph	(De Wergifosse et al., 2004; Inoue et al., 1975)
8	Ph	H	CH_3	(Devillers et al., 2001; Hirano et al., 1993)
9	Ph*p*OCH_3	H	CH_3	(Devillers et al., 2001; Hirano et al., 1993)
10	Ph	H	Ph	(Devillers et al., 2001; Hirano et al., 1993)
11	Ph*p*OH	H	CH_3	(Devillers et al., 2001; Hirano et al., 1993)
12	Ph*p*OH	H	Ph	(Devillers et al., 2001; Hirano et al., 1993)
13	Ph*m,p*$(OH)_2$	H	CH_3	(Burton et al., 2003; Cavalier et al., 2001a, 2001b)
14	Ph*m,p*$(OH)_2$	Ph*p*OH	CH_3	(Burton et al., 2003; Cavalier et al., 2001a, 2001b)
15	Ph*p*OH	Ph*p*OH	CH_3	(Burton et al., 2003; Cavalier et al., 2001a, 2001b; Jeanjot et al., 2003)
16	Ph*p*OCH_3	Ph*p*OCH_3	CH_3	(Burton et al., 2003; Cavalier et al., 2001a, 2001b)
17	Ph	Ph*p*OH	CH_3	(Burton et al., 2003; Cavalier et al., 2001a, 2001b; Jeanjot et al., 2003)
18	Ph*p*OH	Ph	CH_3	(Burton et al., 2003; Cavalier et al., 2001a, 2001b; Jeanjot et al., 2003)



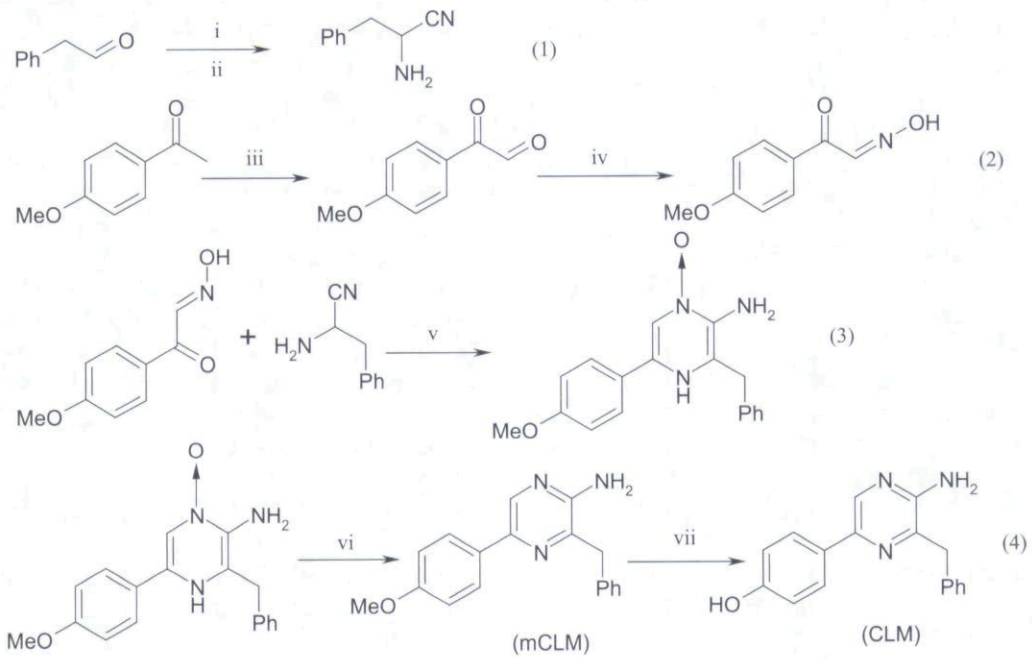
SCHEME 5 First Strategy for the Synthesis of Aminopyrazines (AMPs).

material spontaneously transformed into IMPZ, but in rather low yields (20–40%) (Devillers et al., 2002). Thus, we turned to more traditional synthetic methods.

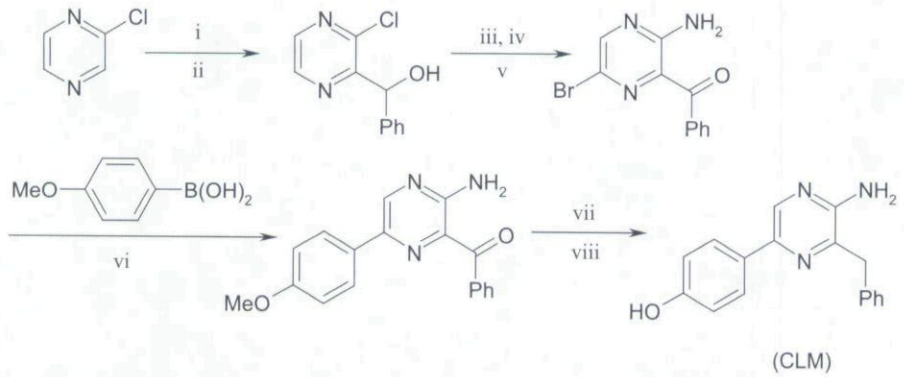
Two strategies of synthesis of 3,7-dihydroimidazolo[1,2-a]pyrazin-3-one derivatives (IMPZs) have been reported in the previous literature (Basiuk, 1997; Porter, 1984). Both are based on the same final step consisting in the cyclocondensation of 2-amino-1,4-pyrazine compounds with appropriate glyoxals (X=H) or α-keto-acids (X=OH) (Kishi et al., 1966). The use of glyoxals (and the corresponding acetals) is preferred because, in this case, formation of the fused five-membered ring occurs in one step (Scheme 4) (Devillers et al., 1999). This heterocyclization was well illustrated with the preparation of ***2*** and ***3***, the simplest IMPZ representatives (Table 1), easily obtained from the commercially available 2-amino-1,4-pyrazine (R^1=R^2=H) and methyl- or phenylglyoxal, respectively, (Devillers et al., 1999, 2001).

The two strategies differ by the mode of construction of the pyrazinyl heterocycle. In the classical route, initially described by Kishi et al. (1966), the pyrazine nucleus was formed by reaction of a 1,2-dicarbonyl compound with a α-amino-amidine derivative (Scheme 5, Eq. 1) (Inoue et al., 1975; Kishi et al., 1972). This method was further improved by White (Karpetsky & White, 1971, 1973): condensation of a α-oximino-ketone with a α-amino-nitrile gave a *N*-oxide-aminopyrazine which could be easily reduced into aminopyrazine with a good overall yield (Scheme 5, Eq. 2). Accordingly, the total synthesis of CLM described by Inoue et al. (1975) made use of this modified first strategy (Scheme 6). Until 1996, this procedure was the only way used to prepare CLM, and some CLZn analogues (Gonzalez-Trueba et al., 1996; Hirano et al., 1993; Shimomura et al., 1989; Teranishi & Goto, 1990; Usami & Isobe, 1996). Nevertheless, this linear strategy suffers from a lack of flexibility about the possible variety of R^1/R^2 substituents, which have to be chosen in the early step of the total synthesis.

The emergence of organometallic chemistry methods for the coupling of substituents on (hetero)aromatic nuclei allowed the development of a more flexible and convergent strategy for the preparation of



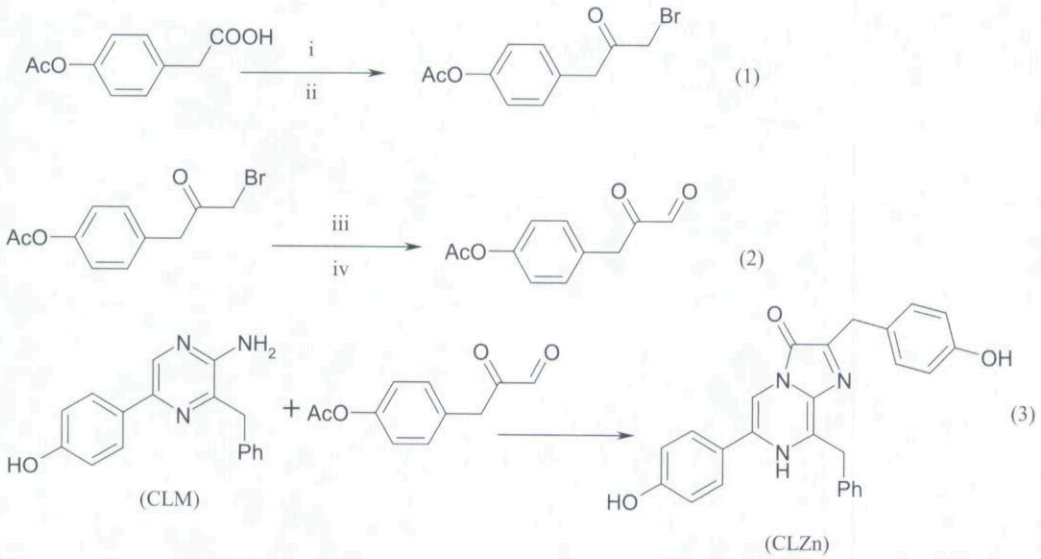
SCHEME 6 Total Synthesis of CLM (First Strategy). Reagents and Conditions: (i) NH_3, EtOH; (ii) KCN; (iii) SeO_2, H_2O-Dioxane; (iv) Me_2C=N(OH), H_3O^+; (v) $TiCl_4$, Pyridine, 80°C; (vi) H_2, Ni-Raney; (vii) Pyridine.HCl, 200°C.



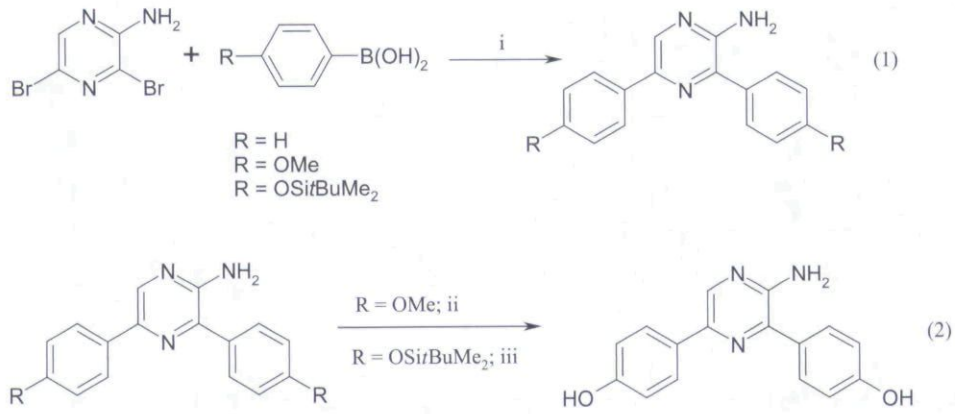
SCHEME 7 **Total Synthesis of CLM (Second Strategy). Reagents and Conditions: (i) LiTMP, THF; (ii) PhCHO; (iii) MnO_2, THF; (iv) NH_3, EtOH, 120°C; (v) Br_2, AcOH, Na_2CO_3; (vi) Pd(dppb)Cl_2 Catal., Na_2CO_3, EtOH, Toluène, Δ; (vii) EtSNa, DMF, 100°C; (viii) NH_2-NH_2, KOH, $(HOCH_2)_2$, 240°C.**

CLM. This second synthesis, initially proposed by Jones (Jones et al., 1996, 1999; Keenan et al., 1997), is summarized in Scheme 7: the R^2 and R^1 substituents were sequentially introduced on the commercially available 2-chloro-1,4-pyrazine. Further coupling of CLM with *p*-(acetoxy)benzylglyoxal (Scheme 8) furnished ***1*** (CLZn), the natural chromophoric substrate of luciferases. The glyoxal derivative has been obtained in four steps from *p*-(acetoxy)phenylacetic acid (Scheme 8) (Gonzalez-Trueba et al., 1996). Condensation of CLM with methyl- and phenylglyoxal afforded respectively ***4*** and ***5***, two CLZn analogues in which R^3 is replaced with CH_3 and Ph (Table 1). Similarly, mCLM (CLM in which the phenol moiety is protected as the methyl ether; see Scheme 6) reacted with methyl- and phenylglyoxal to give ***6*** and ***7***, respectively (Table 1).

The key-step of Jones' synthesis of CLM (Scheme 7, step 6) was the organometallic coupling of *p*-(methoxy)phenylboronic acid on the 5-bromo-2-amino-1,4-pyrazine precursor by using the MacKillop palladium catalyst (Ali et al., 1992). The same strategy could be used for the introduction of non-natural R^1/R^2 substituents from the corresponding boronic acids (Suzuki coupling) (Miyaura & Suzuki, 1995) or *tri*-butyltin compounds (Stille coupling) (Stille, 1986), the mainly considered substituents being aryl, vinyl, and propargyl groups (Nakamura et al., 1995, 2000). As mentioned above, CLZn derivatives were traditionally studied for their remarkable bioluminescent properties (Hirano et al., 1993; Isobe et al., 1998; Ohmiya & Hirano, 1996; Teranishi et al., 1994; Usami & Isobe, 1996). The development of their antioxidative potential in the medicinal chemistry context required the



SCHEME 8 **Total Synthesis of CLZn According to Gonzales-Trueba (1996). Reagents and Conditions: (i) $SOCl_2$; (ii) CH_2N_2 then HBr; (iii) $AgNO_3$, CH_3CN; (iv) AcONa, DMSO; (v) HCl, EtOH-H_2O, 80°C.**

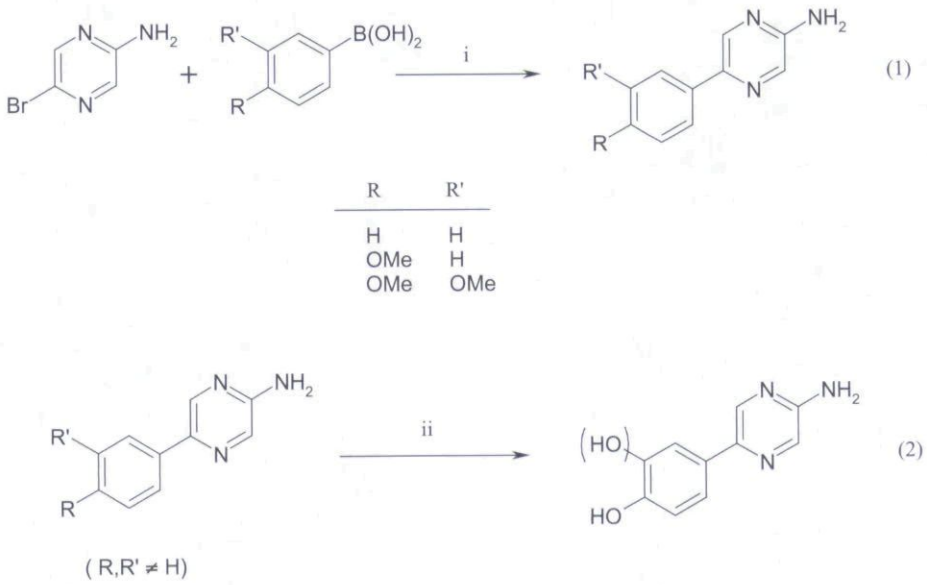


SCHEME 9 **Preparation of Symmetrically-bis-Substitued Aminopyrazines. Reagents and Conditions: (i) $PdCl_2$(dppb) Catal., Toluène, Reflux; (ii) EtSH, $AlBr_3$, 20°C or EtSNa, DMF, 100°C; (iii) Bu_4NF, THF, 0°C.**

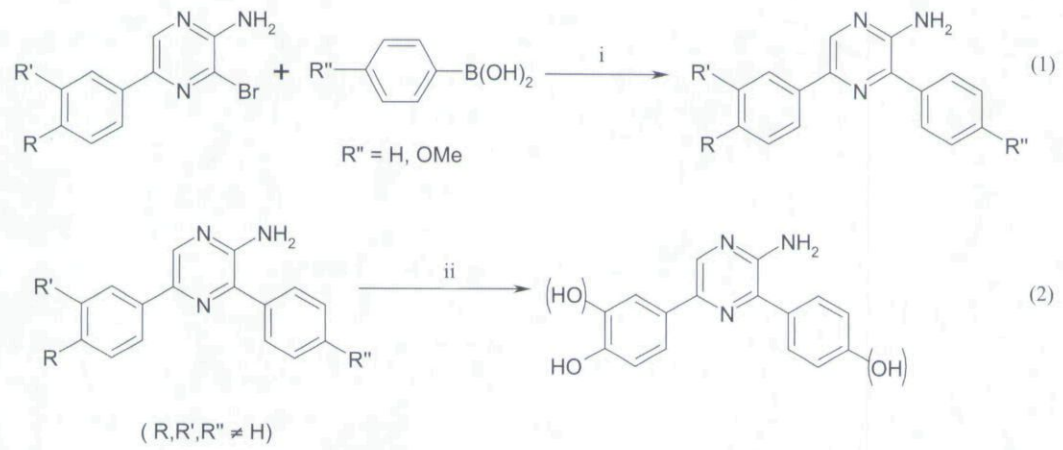
synthesis of novel compounds bearing R^1/R^2 substituents susceptible to improve the biological properties. We thus selected phenyl groups (for extended conjugation) (Wu et al., 2001) equipped with one or two hydroxyl functions (for phenolic radical formation and metal ligand property in the case of a catechol motif).

Starting from 2-amino-3,5-dibromo-1,4-pyrazine, we prepared symmetrically-substituted 2-amino-3,5-*bis*-aryl-1,4-pyrazine derivatives via the Suzuki coupling reaction (Burton et al., 2003; Cavalier et al., 2001a, 2001b) (Scheme 9). Introduction of the phenol motifs required their protection as the corresponding methyl ethers (Eq. 1); accordingly, in a next step, hydroxyl-free derivative was obtained by treatment with ethanethiol and aluminium trichloride (Eq. 2), a classical method for aryl alkyl ether cleavage. However, this procedure requires hard experimental conditions and is not really safe and practical. Recently, we disclosed an alternative procedure based on the *t*-butyldimethylsilyl protecting group which was easily removed by smooth treatment with *tetra*-butylammonium fluoride (Jeanjot et al., 2003) (Scheme 9). The last improvement of CLZn ***1*** synthesis in 2003 made use of this protecting group (Adamczyk et al., 2003).

Unsymmetrically-substituted 3,5-*bis*-aryl-aminopyrazines were obtained from 5-monosubstituted aminopyrazines, themselves resulting from the Suzuki coupling of an arylboronic acid with 2-amino-5-bromo-1,4-pyrazine (Burton et al., 2003; Cavalier et al., 2001a; Jeanjot et al., 2003; Kuse & Isobe, 2000) (Scheme 10, Eq. 1). Bromination of 5-aryl-2-amino-1,4-pyrazine derivatives furnished the precursors of the second Suzuki coupling (Scheme 11, Eq. 1) (Burton et al., 2003; Cavalier et al., 2001a). Lastly, deprotection of the phenolic ethers gave the hydroxyl-free derivatives (Eq. 2 in Schemes 10 and 11) used for



SCHEME 10 **Preparation of Mono-Substitued Aminopyrazines. Reagents and Conditions: See Scheme 9.**



SCHEME 11 **Preparation of Unsymmetrically-*bis*-Subsitued Aminopyrazines. Reagents and Conditions: See Scheme 9.**

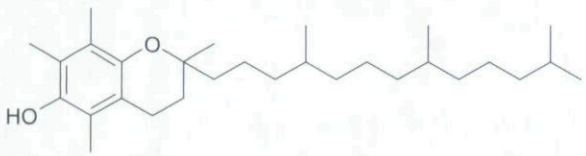
the synthesis of the corresponding IMPZs by cyclocondensation with methyl- or phenylglyoxal (see Scheme 4; X=H; R_3=Ph, Me) (Adamczyk et al., 2001).

Representative IMPZ derivatives used for testing in the next sections are collected in Table 1. The antioxidant potential of CLZn ***1*** and related IMPZs has been determined by several chemical reactivity assays using ROS and reactive nitrogen species (RNS). After that, the molecules were evaluated in acellular and cellular systems representative of pathological conditions, and finally in vivo against ischemia-reperfusion injury.

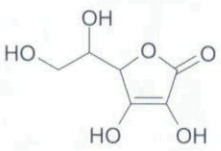
TABLE 2 **Rate Constants of the Reaction of IMPZs with $^1O_2$**

Compound (see Table 1)	k ($\times 10^8$ M^{-1} s^{-1})
1 (CLZn)	2.45
4	16.9
5	28.7
6	25.4 (lit.29.4) (Mashiko et al., 1991)
7	8.6
8	6.3 (Fujimori et al., 1998; Suzuki et al., 1990)
α-tocopherol (a)	6.7
Ascorbic acid (b)	1.6
NaN_3	2.2

(a) Structure of α-tocopherol (vitamin E):



(b) Structure of ascorbic acid (vitamin C):



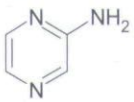
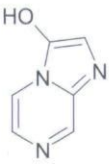
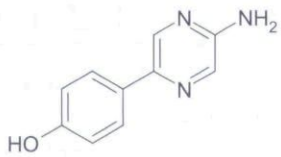
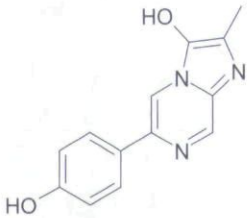
CHEMICAL REACTIVITY ASSAYS

An antioxidant is defined as "any susbstance that when present at low concentrations, compared to those of an oxidizable substrate, significantly delays, or inhibits oxidation of that substrate" (Halliwell, 1995; Halliwell et al., 1995). Biologically relevant oxidants are singlet oxygen, superoxide anion, peroxynitrite, and species derived from the reaction of carbon-centered radicals with molecular oxygen. Tradtionally, the reactivity of a compound towards ROS (or RNS) was considered as the first good indication of its therapeutic antioxidative potential. About 15 years ago, Bensasson et al. established a correlation between the reactivity of imidazoles and phenols with singlet oxygen and their inflammatory and anti-cancerous activities, respectively (Bensasson & Rougée, 1998; Bensasson et al., 1992).

Reactivity of Coelenterazine and Analogues Towards Singlet Oxygen

Singlet oxygen is a ROS involved in photosensibilization processes. In the laboratory, $^1O_2$ is produced by laser excitation (at 532 nm) of Rose Bengal in methanol used as sensitizer for energy transfer to $^3O_2$. Light is emitted at 1268 nm when $^1O_2$ decays to $^3O_2$; this allows $^1O_2$ detection in spite of its very short half-life (223 μs in CD_3OD) (Khan, 1980). In the presence of an antioxidant, the kinetics of $^1O_2$ quenching can be measured taking into account the competitive natural decay.

TABLE 3 ΔE_{stable} Values[a]

Entry	Molecular fragment	ΔE_{stable} values (kcal mol^{-1})
1		stable
2		0.742
3		4.750
4		4.451
5		5.601

[a]All the geometry optimizations and instability calculations have been performed at the ab initio level using the 6-311G** basis set. The ΔE_{stable} values are the energy differences between the singlet state energy and the stabilized one (Dive, G., unpublished results).

We have determined the rate constants of the reaction of IMPZs with $^1O_2$ (Ghislain et al., 1995; Rees et al., 1998). Comparatively to α-tocopherol and ascorbic acid (Bielski et al., 1985), CLZn ***1*** and analogues showed similar or higher reactivities, with a range of k comprised between 2.45 10^{-8} to 2.87 10^{-9} M^{-1} s^{-1} (Table 2).

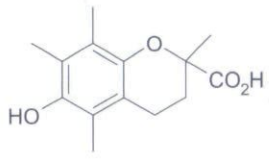
None of the R^1, R^2, and R^3 substituents (see Table 1) influenced dramatically on the reactivity: the phenol motif of R^1 can be masked or suppressed (***6***, ***7***, or ***8***), R^2 can be suppressed (***8***), and R^1 (*p*-hydroxy-benzyl in natural CLZn ***1***) can be replaced with methyl or phenyl (***4***, ***5***). The IMPZ core (R^1=R^2=R^3=H) seems to be intrinsically responsible for ROS quenching. This property has been confirmed by theoretical calculations based on Hartree-Fock wave function instabilities (Dehareng & Dive, 2000; Dehareng et al., 2002). In this approach, an analysis of the propension of the neutral compounds (IMPZs) with all their paired electrons to generate radicular structures is concerned. By their fused five-membered ring, IMPZ derivatives break down the aromaticity and only one Kekule form can be drawn. This feature, the source of the wave function instability, is related to the vicinity of a triplet electronic state near the fundamental singlet state. Imidazole wave function is stable and that of 1,4-pyrazine presents the same weak instability as benzene and pyridine; but the association of the two fragments leads to a significant instability (Table 3). This one can be seen as the ability of the parent molecule (R^1=R^2=R^3=H) to present a local biradicular character and therefore, a propension to be involved into redox processes (Devillers et al., 2001). Interestingly, 2-aminopyrazine substituted with a phenol motif (Table 3, entry 4), also showed Hartree-Fock wave function instability (Dubuisson et al., 2004), and similar substitution on the IMPZ core enhanced the effect (entry 5) comparatively to the parent compound (entry 3).

TABLE 4 Rate Constants of the Reaction of IMPZs with $O_2^{-\cdot}$

Compound (see Table 1)	k ($\times 10^4$ M^{-1} s^{-1})[a]
1 (CLZn)	11.5 (Devillers et al., 1999)
2	6.0 (Devillers et al., 1999)
4	14.7
5	18.1
6	26.3
7	21.5
8	10.6 (Gotoh & Niki, 1994)
9	28.0
11	21.0
13	7.0
14	6.9
15	33.0
16	4.8
17	3.9
18	16.0
Trolox[b]	1.7

[a]The experiments were performed at 25°C in phosphate buffer (pH 7.4).

[b]Structure of Trolox:



Experimentally, CLM (the aminopyrazine daughter-product of oxidative degradation of CLZn ***1***, see Scheme 1) was about ten-fold less reactive than CLZn ***1*** towards singlet oxygen [$k_{CLM}=5.2\pm0.6\times10^{-7}$ M^{-1} s^{-1} Ghislain et al., 1995].

Reactivity of CLZn and Analogues Towards the Radical Anion Superoxide

The radical anion superoxide ($O_2^{-\cdot}$) is a prime ROS formed in all cells by respiratory and other oxido-reduction processes. This free radical can be generated in vitro enzymatically with a xanthine/xanthine oxidase system (Gotoh & Niki, 1992). Thanks to the ability of CLZn ***1*** and some other IMPZs to luminesce upon reaction with $O_2^{-\cdot}$, it is possible to easily perform competition studies with trolox, a commonly used analogue of vitamin E, whose rate constant with $O_2^{-\cdot}$ is known (1.7×10^4 M^{-1} s^{-1}). In that case, the light production resulting from IMPZ reaction with $O_2^{-\cdot}$ decreased as trolox concentration increased, allowing the determination of IMPZ rate constants with $O_2^{-\cdot}$ (Devillers et al., 2001). When IMPZ compounds were non-luminescent with $O_2^{-\cdot}$, these were put in competition with a luminescent IMPZ for which the rate constant was previously determined. All tested IMPZs reacted very rapidly with $O_2^{-\cdot}$ and all the rate constants were superior to that of trolox (Table 4): the values ranged from 3.9×10^4 to 33.0×10^4 M^{-1} s^{-1}. The IMPZ motif seems to be responsible for superoxide scavenging, with a modest influence of the R^1, R^2, and R^3 substituents. Indeed, the reactivity of the natural product ***1*** was only about twice that of ***2*** which presents the naked bicyclic core. The natural R^3 substituent could be replaced by

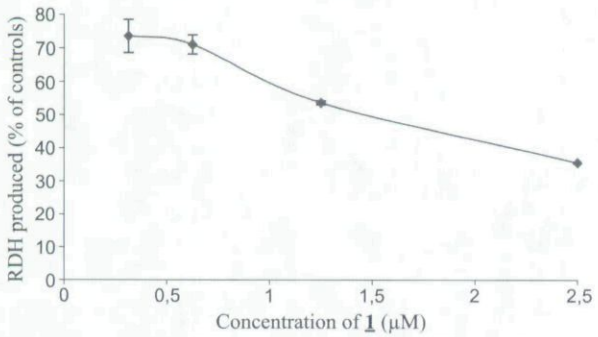


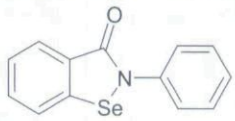
FIGURE 1 Effect of Increasing Concentrations of Celenterazine *1* on $ONOO^-$-Induced DHR Transformation Into RDH. Results are Expressed in % of Controls (Means±SEM of Three Independent Experiments).

TABLE 5 Reactivity of IMPZs (and AMPs) Towards Peroxynitrite

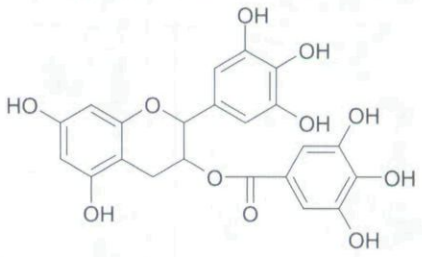
Compound (see Table 1)	IC_{50} (μM)[a]
1 (CLZn)	1.467±0.031 [0.92±0.02 (CLM)]
2	0.290±0.001
3	0.381±0.020
4	1.407±0.003 [0.92±0.02 (CLM)]
5	0.658±0.007 [0.92±0.02 (CLM)]
6	0.953±0.026
7	1.620±0.026
8	0.285±0.009
9	0.356±0.066
10	0.594±0.006
11	0.466±0.006 (0.74±0.03)
12	0.552±0.003 (0.74±0.03)
13	0.433±0.033 (0.53±0.01)
14	0.513±0.026 (0.47±0.01)
15	0.607±0.009 (0.52±0.02)
16	0.816±0.014
17	0.689±0.028 (1.42±0.02)
18	0.516±0.014 (0.50±0.06)
Ebselen[b]	0.94±0.09
Trolox	0.47±0.01
EGCG[c]	0.35±0.03

[a]Inhibition (%) was calculated from the equation $I(\%)=100-A_{inh}/100\ A_o$, where A_{inh} is the signal generated in the presence of the inhibitor (at 2.5 μM) and A_o is the signal observed in the absence of inhibitor. By varying the inhibitor concentration from 0.25 μM to 2.5 μM, a IC_{50} value was determined. This corresponds to the antioxidant concentration giving 50% inhibition of DHR oxidation by peroxynitrite.

[b]Structure of Ebselen (2-phenyl-benzo[d] isoselenazol-3-one):



[c]Structure of EGCG (epigallocatechin-3-gallate) (Zhu et al., 2004):



methyl or phenyl without loss of activity (***4***, ***5***). The phenol group of R^1 could be suppressed (***8***, ***17***), or masked as the methyl ether (***6***, ***7***, ***9***, ***16***) to furnish even more reactive compounds than the reference ***1***. The R^2 substituent could be suppressed (***11***) or

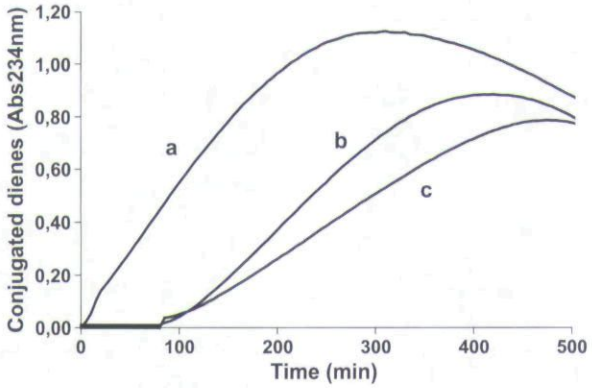


FIGURE 2 **Typical Profiles of AAPH-Induced Linoleate Peroxidation in the Presence or Absence of IMPZ Antioxidants. Lipid Peroxidation is Measured Through the Formation of Conjugated Dienes Measured by Absorbance at 234 nm. Curve a is the Profile Obtained with the Control Sample Treated with AAPH Only. Curve b Shows a Lag Time But No Decrease in the Propagation Rate (Slope) of the Lipid Peroxidation Reaction. Curve c Combines a Lag Time and a Decrease in the Rate of Propagation.**

replaced by a phenyl (***18***) or a supplementary phenol group (***15***); in this last case, the rate constant was maximum (k=33. 10^4 M^{-1} s^{-1}). Finally, the introduction of a catechol motif in R^1 position (***13***, ***14***) did not enhance the reactivity towards $O_2^{\cdot-}$.

This property of a high reactivity with ROS had already been exploited in analytical biochemistry: ***8*** and ***9*** are commonly used tools for detecting superoxide in acellular and cellular systems (Akutsu et al., 1995; Fujita et al., 1995; Gotoh & Niki, 1992; Nakano, 1998; Shimomura et al., 1998).

In contrast to ***1*** and IMPZ analogues, the corresponding aminopyrazines (see Scheme 1, CLM, and related daughter-compounds) were totally unreactive towards $O_2^{\cdot-}$ (Dubuisson et al., 2004).

Reactivity of Coelenterazine and Analogues Towards Peroxynitrite

Peroxynitrite is a product of the reaction between $O_2^{\cdot-}$ and NO (Radi et al., 2002); this RNS behaves as a strong oxidant, in particular towards tyrosine residues of proteins leading to enzymes inactivation and human disorders (Yamakura et al., 1998). It was previously shown that coelenterazine ***1*** emits light upon reaction with ONO_2^- (Tarpey et al., 1999). This prompted us to investigate the reactivity of ***1*** and IMPZ analogues towards pure peroxynitrite synthesized from solid KO_2 and NO gas according to Koppenol et al. (1996). The assay was based on the oxidation of dehydro-rhodamine-123 (DHR) induced by peroxynitrite into fluorescent rhodamine-125 (RDH) which production was followed at 515 nm (excitation wavelength) and 555 nm (emission wavelength) (Bryk et al., 2000). In the presence of IMPZs, quenching of ONO_2^- provoked a decrease of fluorescence.

All tested IMPZs inhibited the DHR transformation in a dose-dependent manner exemplified in Fig. 1 for the natural CLZn ***1***. The IMPZ concentrations required for inhibiting by 50% the DHR (IC_{50}) are listed in Table 5. As previously, the reactivity towards ONO_2^- mainly resided in the bicyclic core with modest influence of the R^1, R^2, and R^3 substituents. As a matter of fact, the unsubstituted compounds ***2*** and ***3*** were among the highest reactive IMPZs; the natural compound ***1*** and very similar derivatives (***4***, ***7***) were less reactive. Some daughter compounds

TABLE 6 **Inhibition of Lipid Peroxidation by IMPZs (Compounds Tested at 5 μM)**

Compound (see Table 1)	Latency time (min)	Inhibition (%)
2	133.54±3.32	0.0±0.52
4	102.15±5.66	45.65±1.47
8	128.14±3.64	2.04±4.89
9	120.94±1.94	3.12±1.53
11	113.31±3.73	30.44±1.51
13	360.91±3.89	31.10±0.63
14	365.91±17.46	35.36±4.32
15	136.95±0.67	63.39±8.38
16	147.74±3.42	11.79±1.74
17	119.01±0.54	28.53±0.69
18	138.25±3.00	53.76±1.05
Vitamin E	252.68±13.23	4.78±3.01
Trolox	120.17±4.05	0.0±1.04
EGCG (Chen et al., 2001)	365.91±17.46	21.21±3.13

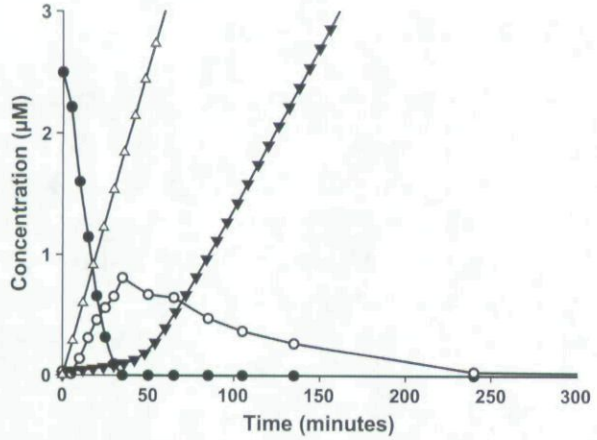


FIGURE 3 **The Antioxidative Cascade. Compound *5* (2.5 μM) was Incubated at 37°C in the Presence of Linoleate and AAPH at 2 mM. The Concentrations of *5* (Filled Circles) and CLM (Open Circles) were Measured in Samples by HPLC. Concurrently, the Concentration of Conjugated Dienes (Filled Triangles) was Monitored Spectrophotometrically at 234 nm. The Curve Made-Up with Open Triangles Corresponds to the Uninhibited Peroxidation Process, in the Absence of *5*.**

corresponding to IMPZs bearing phenol/catechol substituents (namely CLM and related aminopyrazine (AMPs); see Scheme 1) were also evaluated in this peroxynitrite assay. The results are given in parenthesis in Table 5 (Dubuisson et al., 2004). All AMPs were similarly reactive; this could be due to their radical scavenger properties linked to the phenol/catechol sbustituent (the absence of such a substituent led to inactive AMPs; results not shown). Comparatively to classical references such as vitamins E and C, Ebselen, Trolox, and EGCG, CLZn ***1*** and other IMPZs showed roughly the same reactivity towards ROS (Table 5) or a higher reactivity (Tables 2 and 4), but not exceeding twenty-fold.

Inhibition of Lipid Peroxidation by CLZn and Analogues

Lipids are important targets for ROS and their peroxidation can have injurious consequences by causing leaks in cytoplasmic cell membranes. This oxidation process consists in a set of radicalar chain-reactions initiated by hydrogen abstraction from allylic methylene groups of the polyunsaturated fatty acids (Girotti, 1998).

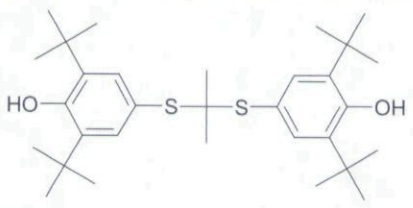
In order to investigate the reactivity of CLZn ***1*** and other IMPZs towards lipid-derived radicals, the AAPH (2,2′-azo-*bis*(2-amidinopropane)dihydrochloride)

TABLE 7 **Protection of LDL Against Oxidation (Compounds Tested at 1.25 μM)**

Compound (see Table 1)	THP (min)[a] $CuSO_4$ (10 μM)	THP (min)[a] AAPH (2 mM)
(Control)	104.44±0.45	244.54±6.16
1	155.12±2.37	277.35±5.25
4	141.86±0.52	234.75±10.29
5	186.90±3.09	313.78±8.08
11	151.67±1.39	275.14±10.75
12	140.81±0.39	284.32±15.02
13	168.61±0.87	306.52±9.85
14	199.12±7.25	233.91±9.25
15	192.02±1.70	342.80±14.25
17	133.80±1.43	327.55±6.41
18	167.39±1.99	199.01±10.60
Trolox	148.57±1.04	—
EGCG	132.47±24.22	—
Probucol[b]	206.79±3.01	—

[a]THP=time of half maximum peroxidation. Cu^{2+} or AAPH-induced LDL oxidation was followed spectrophotometrically at 234 nm (conjugated dienes). Each value is the mean (±SEM) of three replicates.

[b]Structure of probucol (Goodman and Gilman's The Pharmacological Basis of Therapeutics Eight Edition, 1990):



induced linoleate peroxidation system was used (Burton et al., 2003). Micelles were obtained from potassium linoleate and Tween 20 vortexed in borate buffer. Addition of AAPH, a water-soluble free radical initiator, caused a constant production of conjugated dienes by air oxidation, which was monitored at 234 nm. In the presence of an antioxidant, two phenomena could be observed: 1) a latency time or lag phase (thus, the tested compound delayed the onset of AAPH-induced lipid peroxidation); 2) a diminution (inhibition) of the propagation rate calculated as follows:

$$\text{Inhibition}\,(\%) = 100 - R_{inh}/100\ R_o$$

where R_{inh} is the rate of linoleate oxidation in the presence of tested compound and R_o is the rate of linoleate oxidation in the absence of antioxidant. Typical kinetic profiles are shown in Fig. 2, and representative kinetic parameters are collected in Table 6 (Burton et al., 2003). All IMPZs caused very similar latency times (comparable to that of Trolox), comprised between 102 and 148 min, except the catechol derivatives (***13***, ***14***) which provoked higher latency times of 360–365 min (comparable to those of EGCG and vitamin E). Here again, the nature of the R^1, R^2, and R^3 substituents did not greatly influence the lag time (***2***, ***4***, ***8***, ***9***, ***11***, ***15***, ***16***, ***17***, ***18***).

On the other hand, the nature of R^1 was found to be responsible for the inhibition of the propagation rate (curve ***c*** in Fig. 2); the highest acitivities corresponded to R^1 being phenol (***4***, ***11***, ***15***, ***18***) or catechol (***13***, ***14***). When R^1 was hydrogen (***2***), phenyl (***8***) or *p*-methoxy-phenyl [***9*** (Tampo et al., 1998), ***16***] we observed practically no effect on the propagation rate; thus, after the latency period (corresponding to the consumption of the antioxidant), the linoleate oxidation proceeded at a rate similar to that of the control (curve ***b*** in Fig. 2). Interestingly, when R^2 was also a phenol motif, the activity was enhanced (***15***) or restored (***17***). These results suggested that some IMPZ oxidation products could also be endowed with chain breaking properties. Coelenteramide (CLD, see Scheme 1) was devoid of antioxidant properties (Burton et al., 2003; Dubuisson et al., 2001), while CLM (see Scheme 1) behaved as an antioxidant (De Wergifosse et al., 2004, unpublished results) (% inhibition of linoleate peroxidation=61.50+1.11 at 5 μM and 39.26+0.31 at 2.5 μM). We further established that the capacity of CLM to scavenge lipid radicals was linked to the 5-*p*-hydroxyphenyl-2-amino-1,4-pyrazine moiety susceptible to form a highly stabilized phenoxyl radical (Dubuisson et al., 2004).

We have now unambiguously demonstrated that the effect caused by some IMPZs on the lipid peroxidation propagation rate is indeed due to the action of AMP derivatives formed in situ, after consumption of IMPZs with lipid radicals (De Wergifosse, unpublished results). In the experiment described in Fig. 3, the CLZn analogue ***5*** (2.5 μM) was incubated in the presence of linoleate micelles and AAPH. Species evolution was followed in parallel, spectrophotometrically, and by HPLC. It clearly appeared that the production of CLM (see Scheme 1) increases while the concentration of the mother-compound ***5*** decreases. The concentration of CLM (daughter-compound) reached the maximum value when ***5*** was totally consumed. This corresponded to about 40 min, the latency period observed by UV for the conjugated dienes production. After that, CLM was slowly degraded while the rate of linoleate peroxidation was inhibited comparatively to the control. Thus, the produced AMP plays an important role in the chain-breaking properties of the mother IMPZ, provided that the R^1 substituent is a phenol (or catechol) motif; the AMP unmasked on the site of the oxidative stress prolongs the antioxidant action of the IMPZ. Such a mode of action in cascade is unprecedented.

LDL PROTECTION

Cardiovascular disease with plaque formation constitutes a large part of the total burden of diseases in western countries. Oxidative modification of low density lipoproteins (LDLs) is a prerequisite for macrophage uptake, transformation into foam cells and cellular accumulation of cholesterol, causing the formation of early atherosclerotic lesions (Chisolm & Steinberg, 2000; Heinecke, 1998). Therefore, decrease of LDL oxidation is an important strategy in atherosclerosis prevention. There are numerous studies examining the effects of antioxidants on the ex vivo oxidation of LDLs (Jeong et al., 2004; Lee et al., 2004). Recent data reinforced the concept that a regular intake of antioxidants present in food blocks the

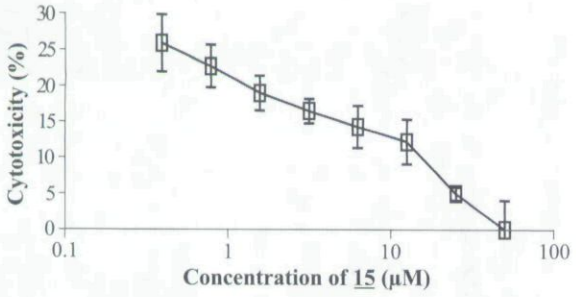


FIGURE 4 **Effect of Increasing Concentrations of Compound *15* on the Survival of UVB-Irradiated Keratinocytes (200 mJ/cm^2; 3 min). Cytotoxicity was Estimated from LDH Release in the Culture Medium 24 Hours After Irradiation. Results are Expressed as % of Survival Relatively to Non-Irradiated Control Cells (Mean ± SEM of Six Replicates).**

progression of atherosclerosis, and that the reduced ability of LDLs to oxidize may represent a good marker to follow the action of antioxidants (Norata et al., 2003). As IMPZs showed excellent ability to delay and slow down lipid peroxidation, we studied their potential protective effect on LDLs submitted to oxidants.

Human LDLs were prepared as previously described (Dubuisson et al., 2004). One of the most common techniques for initiating LDL oxidation in vitro involves incubation with Cu^{2+} (Gaetke & Chow, 2003) and spectrophotometric monitoring of the formation of conjugated dienes (Esterbauer et al., 1989). Coelenterazine ***1*** at 1.25 μM protected LDL against copper-induced oxidation, as shown by the increased time of half peroxidation (THP) around 50%. The comparison with analogues indicated that the activity could be slightly modulated by structural modification as shown in Table 7.

The natural R^3 substituent (*p*-hydroxybenzyl) could be replaced with methyl (***4***) or phenyl (***5***) without loss of activity. The natural R^2 substituent (benzyl) could be suppressed (***11***, ***12***, ***13***). On the other hand, the presence of at least one phenol substituent, preferably at R^1, appeared essential for activity (***15***, ***18***, versus ***17***). A catechol motif increased the activity (***13***, ***14***) and the most active compounds (***14***, ***15***) displayed two phenol/catechol motifs in the R^1/R^2 positions. Protected compounds, namely methoxylated derivatives ***9*** and ***16***, were not active at 1.25 μM (results not shown). Coelenterazine ***1*** and analogues ranged in the activity domain of the natural antioxidant EGCG and the synthetic compound Probucol usually considered as a reference for the reduction of total plasma cholesterol (Goodman & Gilman's The Pharmacological Basis of Therapeutics Eight Edition, 1990).

Protection of LDLs against Cu^{2+} induced oxidation could proceed both through the scavenging of lipid radical intermediates and the formation of inactive chelation complexes with copper. Thus, copper chelation by IMPZs was spectroscopically investigated (modifications of the UV spectra in the prescence or absence of copper sulfate). Most IMPZs with R^3 being an alkyl substituent complexed copper, while no chelation was detected when R^3 was a phenyl substituent (***5***, ***12***). This could be correlated with the predominant enol form (R^3 = Me) over the ketone form (R^3 = Ph) for 2-alkyl IMPZs (Devillers et al., 1999) (see the structures in equilibrium drawn in Table 1). Nevertheless, compounds ***5*** and ***12*** markedly slowed Cu^{2+}-induced LDL oxidation, suggesting that copper chelation is not entirely responsible for the inhibition. This was demonstrated by the ability of the IMPZs to change the kinetics of AAPH-induced LDL oxidation (Table 7). Results obtained with AAPH as inducer of LDL peroxidation differed from those obtained with Cu^{2+}. Indeed, compounds ***1***, ***4***, ***11***, ***18***, and ***14*** (tested at the same concentration as in Cu^{2+}-induced oxidation) did not significantly delay the peroxidation induced by AAPH, indicating that their protection of Cu^{2+}-induced oxidation involved copper chelation. Higher concentrations (results not shown) were required to slow down the peroxidation process suggesting that the inhibition does not solely reside in this Cu^{2+} immobilization effect. On the other hand, ***15***, ***17***, and ***13***, which also chelated copper, remained effective against AAPH-triggered oxidation at 1.25 μM. Finally, ***5*** and ***12***, which

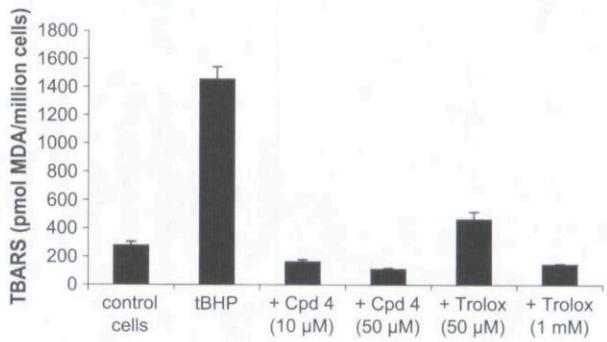


FIGURE 5 **Effect of Compound *4* and Trolox on Lipid Peroxidation Induced by tBHP in Rat Hepatocytes and Measured by the TBARS Test (Mean ± SEM of Three Replicates).**

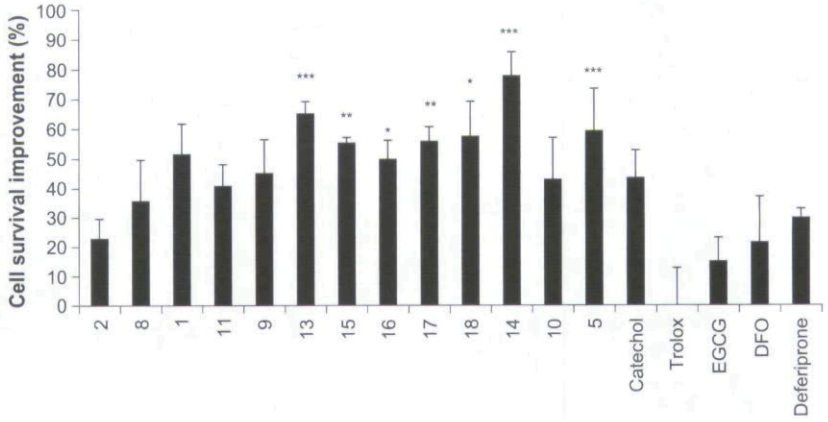


FIGURE 6 Improvement of Cell Survival (% of Total Protection) in PC12 Cells Submitted to 250 μM of H_2O_2 During 24 Hours in the Presence or Absence of 50 μM of IMPZs or Reference Antioxidants. Cell Survival was Measured by the LDH Test. Data are Presented as Mean ± SEM of Three Independent Experiments.

showed no Cu^{2+} chelation, also protected LDLs against AAPH-induced peroxidation at 1.25 μM. Thus, the chelating properties of IMPZs that prevent Cu^{2+} from taking part in redox processes, can add to their chain-breaking action and reinforce their antioxidative potential. Metal chelation is a characteristic also found for polyphenolic antioxidants such as EGCG (Chen et al., 2001).

CELLULAR TESTS

Cellular Toxicity

The potential toxicity of IMPZ derivatives has been evaluated on several cell types (keratinocytes, hepatocytes, fibroblasts, endothelial cells, pulmonary cells, etc.) by measuring cell survival in culture systems containing the tested compounds. Since most IMPZs are hydrophobic, their solubility in culture medium limited the maximal concentration tested at 100 μM. Conventional MTT and LDH assays (see below) were used to estimate cell survival. Interestingly, all the compounds were found to be devoid of toxicity. Only one noticeable exception occurred with the natural compound ***1*** on rat hepatocytes, probably due to interactions at cytochrome P450s level (Dubuisson et al., 2000). Indeed, no such toxicity was observed for CLZn ***1*** in other cell types.

Protection of Human Keratinocytes Against UVB-Irradiation Damage

The epidermis is composed mainly of keratinocytes. Upon UV exposure, photooxidative reactions are initiated, causing damage to biomolecules and affecting

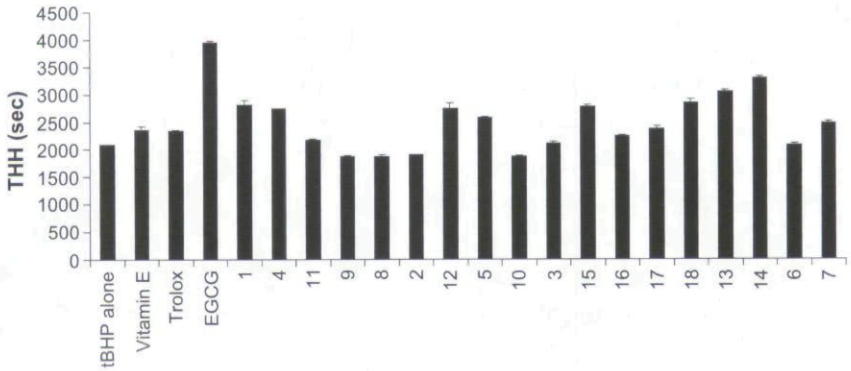
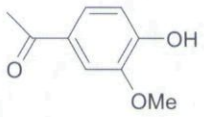


FIGURE 7 Effect of IMPZ Compounds on the Time of Half-Haemolysis (THH) Induced by 500 μM of tBHP in Fish Erythrocytes. All the Antioxidants were Used at a Concentration of 10 μM. Data Represent Mean ± SEM of Three Independent Experiments.

TABLE 8 Protective Effect of IMPZs on Ischemia/Reperfusion Injury

Compound (see Table 1)	% Inhibition (30 mg/kg)	% Inhibition (3 mg/k)	% Inhibition (0.3 mg/kg)
2 (Arrault et al., 2003)	49	—	—
3 (Arrault et al., 2003)	79	48	—
15 (Dubuisson et al., 2004)	85	67	52
17 (Dubuisson et al., 2004)	87	53	50
Apomycin[a]	—	59	—

Leaks inhibition (%).
[a]Structure of Apomycin (acetovanillone):



the integrity of cells and tissues (Berton et al., 1997). These reactions are associated with the generation of ROS, which have been observed in vivo (Yasui & Sakurai, 2000). UVB (280–320 nm) irradiation is considered to be responsible for inducing various skin disorders, including skin cancer (F'guyer et al., 2003). Compounds that can protect against these radiations could consequently be used as photochemoprotective agents (Herzog et al., 2004).

The protective effect of IMPZs has been evaluated on human keratinocytes (HaCaT) preincubated for 30 min with the tested compounds and then irradiated with UVB (312 nm). Fresh culture medium was then added for 24 hours. At that time, cytotoxicity tests were realized by measurements of the activity of the mitochondrial enzyme succinate dehydrogenase (MTT test) (Dubuisson et al., 2000) or by the release of the cytosolic enzyme lactate dehydrogenase (LDH) in the supernatant because of cell membrane loss of integrity (Dubuisson et al., 2004).

As shown in Fig. 4, compound ***15*** protected the cells dose-dependently against 200 mJ/cm^2 UVB irradiation. This effect was independent of any filter effect as the presence of ***15*** during the irradiation was not required for the protection to take place. Similar results were obtained with compound ***4***. In this case, it was also shown that the IMPZ protected HaCaT's nuclear DNA against UVB-induced damage as measured by the COMET assay. Other experiments indicated that the protection of keratinocytes by the "naked" compound ***2*** resulted from a filter effect as no protection took place when this IMPZ was absent during irradiation.

We have previously shown that the AMPs (daughter-compounds) derived from the oxidation of ***15*** and ***4*** (namely CLM), similarly protected HaCaT cells (0% LDH release at 50 μM) (Cavalier et al., 2001b).

Protection of Rat Hepatocytes Against Oxidative Stress Induced by Nitrofurantoin and *tert*-Butyl Hydroperoxide

Primary culture of rat hepatocytes is a very useful model to test antioxidants in vitro. Indeed, hepatocytes are highly exposed to oxidative stress in vivo and a great number of liver pathologies are linked to that (Poli, 1993). Hepatocytes treated either with nitrofurantoin or with *tert*-butyl hydroperoxide (t-BHP) undergo oxidative stress, leading to membrane leakage, lipid peroxidation, and DNA damage. Our results indicated that micromolar concentrations of an IMPZ like ***4*** increased cell survival as compared to control cells. One hundred times more concentrated solutions of reference antioxidants such as Trolox or Probucol had to be used to increase cell survival as efficiently as ***4*** (Dubuisson et al., 2000, 2001).

IMPZs also efficiently protected cellular lipids against peroxidation as measured by the TBARS (thiobarbituric acid reactive substance) assay (Dubuisson et al., 2000, 2001) in tBHP- and nitrofurantoin-treated hepatocytes. In this test, lipid oxidation was monitored by the appearance of malondialdehyde (MDA), one of the final products resulting from the peroxidation of polyunsaturated fatty acids. A chromogenic derivative was formed by reaction with thiobarbituric acid and detected by fluorescence spectroscopy (Wey et al., 1993). Figure 5 showed that compound

4 totally protected the hepatocyte lipids at 10 μM; similar results were obtained with Trolox at 1 mM.

The CLZn analogues yielding CLM as oxidation product more efficiently increased hepatocyte survival, suggesting that the antioxidative cascade observed in lipid peroxidation systems (see p. 839) could occur in cells. However, the experimental proofs that AMP is generated in hepatocytes subjected to oxidants, and that CLM prolongs the antioxidant protection first provided by the mother-compound, are still to be brought.

Protection of Neuronal Cells Against Oxidative Stress Induced by Hydrogen Peroxide

The role of ROS in neuronal cell damage leading to disorders, such as Parkinson's and Alzheimer's diseases has been pointed out (Bebbington et al., 2002; Marciniak & Petty, 1996). The rat pheochromocytoma line PC12 cells provide a useful model system for in vitro investigation of neuronal injury, especially with the use of peroxides (Vimard et al., 1996). Coelenterazine ***1*** and analogues were tested as described elsewhere (Dubuisson et al., 2004) and cell survival was measured by the LDH test (the amount of lactate dehyrogenase released into the cell culture supernatant is an indirect measurement of the damage occurring at the cytoplasmic membrane). We found that IMPZs are powerful protectors of PC12 cells submitted to lethal doses of hydrogen peroxide. For cells treated with 250 μM H_2O_2 during 24 h, the mortality level reached 50%. All IMPZs, tested at 50 μm, protected PC12 cells, but significant diffrerences existed in their neuroprotective activity. As shown in Fig. 6, compounds with a catechol function at R^1 (***13***, ***14***) were the most active IMPZs. The protection they offered was superior to that provided by catechol, suggesting that the heterocyclic core should participate in their mode of action. Other IMPZs bearing a phenol function at R^1 (***1***, ***11***, ***5***, ***15***, ***18***) had a pronounced effect on the cell survival. However, the protective effect was not dependent on the occurrence of this phenol motif, since the methoxylated compounds (***9***, ***16***) were similarly efficient. Compounds with a phenyl such as R^1 substituent were still active (***8***, ***10***, ***17***). The lack of significant activity of ***2*** could be explained by the hydrophilicity of the compound, preventing membrane diffusion.

The very high activity of ***13*** and ***14*** suggested that part of their protective effect could be due to the chelation of transition metals present in cells. Indeed, commonly used metal chelators such as desferrioxamine (DFO) and deferiprone protected PC12 cells (Fig. 6), but their protection extent was lesser than that of most IMPZs. Thus a chelating effect could only explain part of the IMPZ efficiency. Moreover, these results should be taken with care since bioavailability of DFO and deferiprone is probably not as good as for the IMPZs. Nevertheless, chelation appeared to be not essential as ***5*** was endowed with high protective activity in this test and did not chelate metal ions such as iron and copper (see p. 841). Trolox and EGCG were respectively inactive and very poorly active in this test.

Protection of Fish Erythrocytes Against Peroxide-Triggered Hemolysis

This cellular model (Janssens et al., 2002a) relies upon fish erythrocytes subjected to an oxidant, in a way similar to that developed in studies on mammalian erythrocytes (Yamamoto et al., 1986). One advantage of fish erythrocytes is that these cells retain an intact nucleus allowing DNA-damage to be studied in parallel with the COMET assay (Janssens et al., 2002a). In this system, sea bass erythrocytes were submitted to t-BHP and hemolysis was followed by the time-dependent decrease in absorbance at 540 nm. The addition of CLZn ***1*** dose-dependently delayed the onset of red blood cell (RBC) hemolysis induced by t-BHP (Janssens et al., 2002b). A retardation of lipid peroxidation and a protection of cellular GSH stores against oxidation accompanied this effect. Furthermore, DNA damage was markedly reduced by CLZn ***1*** (Janssens et al., 2002b). We have used this simple system for comparing the efficiency of synthetic IMPZs with that of the natural compound ***1***. The retardation action of these compounds is presented in Fig. 7. Compound ***4*** afforded a similar protection to that of ***1***. As in other cell-based tests, compound ***2*** was ineffective in preventing hemolysis, probably due to the low diffusibility of this compound into cells.

Derivatives ***8***, ***9***, and ***6*** were devoid of protective properties at the same concentration of 10 μM. As in other cells, the most efficient IMPZs were the catechol derivatives ***13*** and ***14***. The reference EGCG was highly active in this test.

IN VIVO EVALUATION

The in vivo toxicity of compound ***2*** (the simplest imidazolopyrazinone derivative), administrated orally, has been investigated in mice. Remarkably, no sign of acute or chronic toxicity has been recorded at the highest dose tested (1200 mg/kg!) although the compound was absorbed.

The good bioavailability of IMPZs was demonstrated in the "hamster cheek pouch" assay for in vivo protection against ischemia-reperfusion injury. Fluorescent labelled dextran was injected intravenously to animals and changes in the number of microvascular leaky sites were measured after local ischemia/reperfusion by direct observation on microscope of the cheek pouch. This allowed quantitative studies of microvascular permeability (Bertuglia et al., 1996; Bouskela et al., 1997). Animals were treated by gavage with the tested compounds at 30 mg/kg, 3 mg/kg, and 0.3 mg/kg 30 minutes before anesthesia. Results of Table 8 are given in percentages of inhibition of leaky sites, determined 30 minutes after the start of reperfusion. All tested compounds provided good to excellent protection against the increase of microvascular permeability due to ischemia/reperfusion (Arrault et al., 2003; Dubuisson et al., 2004). The most active compounds (***15***, ***17***) were still efficient at 0.3 mg/kg and more active than Apocynin, a flavonoid usually considered as the reference in this test.

Since the R^1/R^2 unsubstituted IMPZs (***2***, ***3***) were active, we could confirm that the activity is related to the fused heterocyclic structure. However, the intrinsic properties of the IMPZ core could be dramatically enhanced by the addition of phenol (catechol) substituents at the R^1 and/or R^2 positions (***15***, ***17***). We think that this effect is mainly due to the in situ production of phenolic AMP derivatives during the oxidative degradation of IMPZs; such compounds are also endowed with antioxidant activity and thus prolong or increase the effect of their mother-compounds.

Current research strategies for emergency reperfusion therapy involves the use of small molecules that can act as ROS scavengers, but can also gain access to the intracellular compartments, such as vitamin analogues with improved pharmacological profiles (Koufaki et al., 2004). In the hamster model, we have illustrated the high therapeutic potential of IMPZs, but also their good intestinal absorption and metabolic stability, as well as their ability to access to cellular compartments where ROS are produced or diffuse.

CONCLUSION

The study of marine bioluminescence, and molecules responsible for this phenomenon, led us to discover the remarkable antioxidative properties of CLZn ***1*** and analogues possessing the same IMPZ core. These heterocycles reacted very rapidly with various ROS/RNS such as singlet oxygen, superoxide anion, peroxynitrite, and reactive intermediates of lipid peroxidation. In all tests, IMPZs were generally more reactive than the classical references, vitamine E, vitamin C, Trolox, and EGCG.

The propension of IMPZs to be involved into redox processes has been confirmed theoretically by the calculation of Hartree-Fock wave function instabilities.

Coelenterazine ***1*** and analogues also showed high protective effects on human LDLs and various mammalian cells submitted to an oxidative stress. Beside their intrinsic reactivity, the effects of IMPZs probably resulted from a good diffusibility into cells and membranes, an appropriate localization near the sites under oxidative threat, and in some instances, from metal-chelating properties. This last effect could be enhanced by a catechol substituent at R^1.

We have demonstrated in vitro that the oxidation of CLZn analogues generates CLM, an aminopyrazine derivative also endowed with antioxidant activity. This provided a unique antioxidant system working as a cascade which could play a role in the high cellular protection conferred by IMPZs, as well as in the in vivo remarkable efficiency of compound ***15*** ($R^1=R^2=p$-hydrophenyl).

Since the biological activity of IMPZs is essentially associated to the bicyclic core and the presence of one phenol (or catechol) substituent (preferably at the R^1 position), the design of novel analogues with similar

activities but with different physico-chemical properties could be now envisaged by playing with the R^2 and R^3 substituents. The synthetic schemes we have developed are well adapted to such structural modifications of IMPZs, with the aim of modulating their tissue and cellular distribution and therefore their pharmacological performances.

The discovery of antioxidant properties in a molecule first detected in some very peculiar deep-sea organisms points out the interest and the need to further investigate the biochemical and physiological adaptations of these creatures to their extreme environment. Since human investigation has only touched very small percentage of the deep sea, we are bound for more discoveries with potential applications for human health in the future.

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