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Synthesis, Structure–activity Relationship and *In Vitro* Evaluation of Coelenterazine and Coelenteramine Derivatives as Inhibitors of Lipid Peroxidation

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Coelenterazine (2-*p*-hydroxybenzyl-6-(3′-hydroxyphenyl)-8-benzyl-3,7-dihydroimidazolo[1,2-a]pyrazin-3-one,CLZn) and coelenteramine (2-amino-3-benzyl-5-(4′-hydroxyphenyl)-1,4-pyrazine CLM), first described as luciferin and etioluciferin, respectively, of bioluminescent systems in marine organisms are endowed with antioxidant properties. This study was aimed at understanding the structural basis of their chain-breaking properties and at designing new compounds with improved antioxidative properties. For this, a series of 2-amino-1,4-pyrazine derivatives and their related imidazolopyrazinones were synthesised and examined for their capacity to inhibit lipid peroxidation in linoleate micelles subjected to the peroxidizing action of AAPH. Structure–activity relationship studies indicated that the reduction of the peroxidation rate by CLM is mainly determined by the concomitant presence of 5-*p*-hydroxyphenyl and 2-amino groups in para position. The lipophilic character of substituents also affected this effect. All imidazolopyrazinones induced a lag-time before the onset of the peroxidation process. The hetero-bicyclic imidazolopyrazinone moiety appears as the main contributor to this activity while phenol groups play little role in it. On the other hand, phenol groups were required for the reduction of the peroxidation rate after the lag-phase. The introduction of a supplementary *p*-hydroxyphenyl substituent at C_8 position did not increase chain-breaking properties. The substitution of the C_5-*p*-hydroxyphenyl with a catechol moiety or the introduction of a second amino group on the pyrazine ring yielded the most active compounds, superior to imidazolopyrazinones and reference antioxidants like epigallocatechin gallate, vitamin E and trolox. The strong antioxidant properties of 2,6-diaminopyrazines are not dependent on the presence of hydroxyl groups indicating that their reaction mechanism differs from that of 2-amino-1,4-pyrazine derivatives.

Keywords: Antioxidant; Aminopyrazine; Imidazolopyrazinone; Lipid peroxidation

INTRODUCTION

Coelenterazine (2-*p*-hydroxybenzyl-6-(3′-hydroxyphenyl)-8-benzyl-3,7-dihydroimidazolo[1,2-a]pyrazin-3-one, CLZn, Scheme 1, Table II) is the substrate for bioluminescent reactions in many marine animals.[1] CLZn and synthetic analogs such as methyl-coelenterazine (CLZm) have been shown to possess strong antioxidant properties. Indeed, they have been reported to scavenge a wide variety of ROS such as superoxide anion,[2,3] singlet oxygen,[4] and peroxynitrite.[5] They protect rat hepatocytes, human fibroblasts and keratinocytes, and fish red blood cells against lipid peroxidation and cytotoxicity induced by *tert*-butyl hydroperoxide and UVB radiation.[6–9] In a recent study we demonstrated that coelenteramine (CLM), an oxidation product of CLZn and CLZm (Scheme 1, Table I) also protects cells against oxidative damage, thus raising the possibility that these compounds

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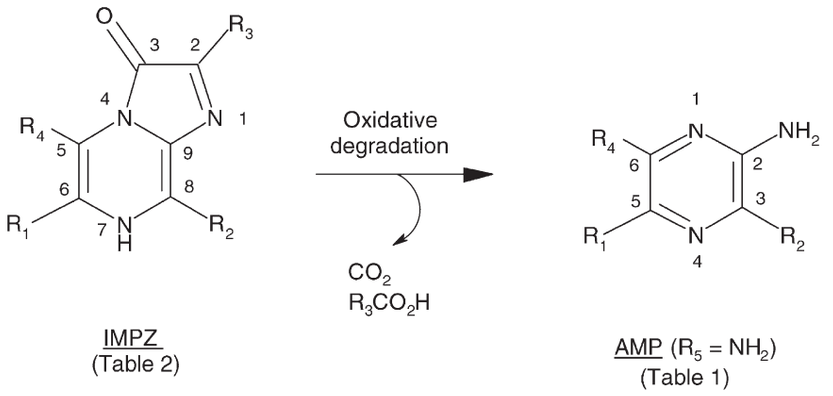


SCHEME 1 Imidazolopyrazinone (IMPZ) and aminopyrazine (AMP) derivatives.

constitute a chain of two antioxidants acting as a cascade.[9,10]

In this paper, we report on the synthesis and antioxidant activity evaluation of a series of CLM derivatives and their corresponding imidazolopyrazinones in an acellular lipid peroxidation assay. Chemical modifications were carried out on CLM and CLZm in order to determine the structural features required for the inhibition of lipid peroxidation by these two antioxidants. Based on this structure–activity relationship study, new aminopyrazine derivatives and their corresponding imidazolopyrazinones were designed. These molecules provided improved antioxidant activities compared to the natural compounds and reference antioxidants.

MATERIAL AND METHODS

Chemical Synthesis

Instruments, Analyses and Materials

Elemental analyses were performed for C, H, N at University College London (UK); the agreement with the calculated data are within ±0.4%, otherwise mentioned. ^{1}H and ^{13}C NMR spectra were recorded

TABLE I CLM and derivatives with their partition coefficients log $P_{octanol/water}$. Log P values (± s.e.m) were calculated on triplicates only on selected compounds. Nd, not determined

Compound	R^1	R^2	R^4	R^5	Log P
CLM	Ph*p*OH	CH_2Ph	H	NH_2	1.09 ± 0.03
1a	Ph*p*OH	H	H	NH_2	0.13 ± 0.01
1b	Ph*p*OH	H	H	H	1.25 ± 0.32
1c	Ph*p*OCH_3	H	H	H	1.37 ± 0.12
1d	Ph	H	H	NH_2	1.85 ± 0.04
1e	Ph*p*OCH_3	H	H	NH_2	1.92 ± 0.03
1f	Ph*o*OH	H	H	NH_2	1.88 ± 0.51
1g	Ph*m*OH	H	H	NH_2	1.58 ± .041
1h	Ph-3,4-diOH	H	H	NH_2	0.94 ± 0.16
1i	Ph-3,4-diOMe	H	H	NH_2	Nd
1j	Ph-2,4-diOH	H	H	NH_2	Nd
1k	Ph*p*OH	H	H	NH–CH_2Ph	>2
1l	Ph*p*OCH_3	H	H	NH–CH_2Ph	0.96 ± 0.04
2a	Ph*p*OH	Ph*p*OH	H	NH_2	1.95 ± 0.09
2b	Ph*p*OH	Ph*p*OH	H	H	>2
2c	Ph*p*OCH_3	Ph*p*OCH_3	H	H	>2
2d	Ph*p*OCH_3	Ph*p*OCH_3	H	NH_2	>2
2e	Ph	Ph*p*OH	H	NH_2	>2
2f	Ph	Ph*p*OCH_3	H	NH_2	>2
2g	Ph*p*OH	Ph	H	NH_2	1.85 ± 0.06
2h	Ph*p*OCH_3	Ph	H	NH_2	>2
2i	H	H	NH_2	NH_2	Nd
2j	Ph*p*OH	Ph*p*OH	NH_2	NH_2	1.47 ± 0.03
2k	Ph*p*OCH_3	Ph*p*OCH_3	NH_2	NH_2	Nd
2l	Ph	Ph	NH_2	NH_2	Nd
2m	Ph*p*OH	Ph-3,4-diOH	H	NH_2	1.10 ± 0.04
2n	Ph-3,4-diOH	Ph*p*OH	H	NH_2	Nd
2o	Ph*p*OH	Ph*p*OH	H	NH–CH_2Ph	>2
2p	Ph*p*OCH_3	Ph*p*OCH_3	H	NH–CH_2Ph	0.96 ± 0.04

on Varian Gemini 200 and Varian Gemini 300 spectrometers; chemical shifts are given in ppm (δ) relative to tetramethylsilane (TMS) as internal standard.

Melting points (not corrected) were measured on an Electrothermal AI-9100 apparatus. The mass spectra, in EI (electronic ionization) and FAB (fast atom bombardment) modes, were obtained on a Finnigan MAT TSQ-70 instrument. Thin layer chromatographies (TLC) were carried out using silica gel $60F_{254}$ plates (0.2 mm, Merck) and the spots were visualized by UV at 254 nm. Column chromatographies were performed on Merck silica gel 60 (70–230 mesh).

Solvents were dried and distilled prior to use. Reagents (Aldrich or Acros) were used as purchased. Several compounds were described elsewhere: **1a**, **1d**, **1e**, **3a**, **3b** and **3c**, in Ref. [11]; **1h**, **1i**, **1j**, **2a**, **2d**, **2m**, **2n**, **3g**, **3j**, and **3l** in Ref. [6]; **3d** in Ref. [18]; **2i**, **2j**, **2k** and **2l** in Ref. [12]; CLZm in Ref. [7].

General Procedures

Functionalization of 1,4-pyrazines (Suzuki coupling reaction). A mixture of *bis*(benzonitrile) palladium (II) dichloride (0.05 equiv./halogen atom to be substituted) and 1,4-*bis*(diphenylphosphino)butane (dppb, 0.06 equiv./halogen atom to be substituted) in dry toluene (2 ml/mmol of pyrazine) was stirred at room temperature under argon atmosphere for 30 min, until a creamy orange slurry of [1,4-*bis*(diphenylphosphino)butane]-palladium (II) chloride was formed. Halogenopyrazine (mono- or dihalogeno-compound), arylboronic acid (1.1 equiv./halogen atom to be substituted), ethanol (0.5 ml/mmol of pyrazine), 1 M aqueous sodium carbonate (1 equiv./halogen atom to be substituted) and toluene (2 ml/mmol of pyrazine) were added to the preformed catalyst and the mixture was heated under reflux for 24 h. Water (5 ml/mmol of pyrazine) was added, and the mixture was diluted with ethyl acetate (6.5 ml/mmol of pyrazine). The aqueous phase was separated and extracted with ethyl acetate (2×6 ml/mmol of pyrazine). The combined organic phases were washed with brine (2×6 ml/mmol of pyrazine), dried over magnesium sulfate, filtered over celite and concentrated under vacuum. The crude product was purified by column-chromatography on silica gel.

N-Benzylation of amino-pyrazines. A mixture of 2-aminopyrazine (1 equiv.), lithium hexamethyldisilylazanate (LiHMDS) (1.5 equiv.) in dry THF (3 ml/mmol of pyrazine) was stirred for 1 h at 20°C. A solution of benzylbromide (1.1 equiv.) in dry THF (3 ml/mmol of pyrazine) was added dropwise. The mixture was stirred for 20 h at 20°C. Ethyl acetate (20 ml/mmol of pyrazine) was added; the solution was washed with 5% aqueous sodium carbonate (2×10 ml/mmol of pyrazine) and brine (3×10 ml/mmol of pyrazine). The aqueous phase was extracted with ethyl acetate (3×10 ml/mmol of pyrazine). The combined organic layers were dried over magnesium sulfate, filtered and concentrated under vacuum. The crude product was purified by column-chromatography on silica gel.

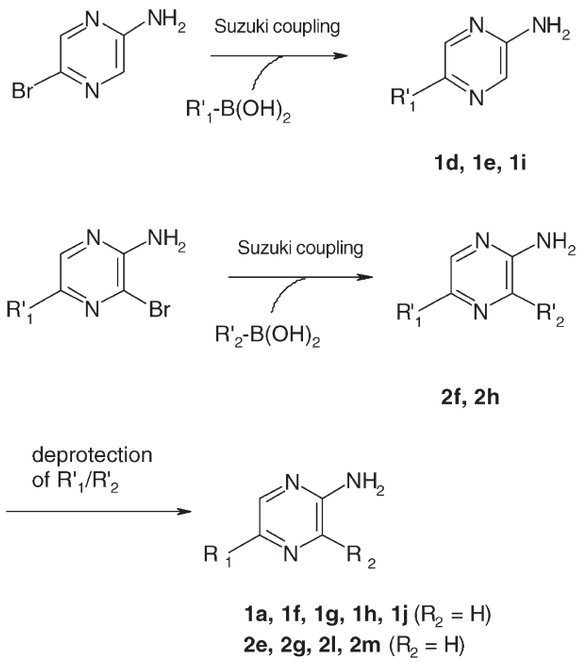
Methyl ether deprotection (method A). A mixture of methoxyphenyl compound (1 equiv.) and sodium ethanethiolate (4 equiv.) in DMF (5 ml/mmol of ether) was heated at 100°C, under stirring, for 24 h. Ethyl acetate (50 ml/mmol) and 10% aqueous ammonium chloride (40 ml/mmol) were added. The organic phase was separated. The aqueous phase was extracted with ethyl acetate (4×30 ml/mmol). The combined organic layers were washed with brine (2×30 ml/mmol), dried ($MgSO_4$), filtered and concentrated under vacuum. The crude phenolic derivative was washed (several times) with a 1:1 mixture of ether and ethyl acetate.

Methyl ether deprotection (method B). To a stirred solution of aluminum chloride (4 equiv./ether function) in ethanethiol (5 ml/mmol of ether), cooled with an ice-water bath, was added the methoxyphenyl derivative in one portion. The mixture was allowed to warm-up slowly to room temperature. After stirring overnight, the mixture was poured into water, acidified with 1 M HCl and extracted with ethyl acetate (3×100 ml/mmol of ether). The organic layer was washed with brine (2×25 ml) and 5% aqueous sodium carbonate (2×25 ml). The aqueous phase was re-extracted with ethyl acetate (2×50 ml). The combined organic layers were washed with brine (2×25 ml), dried over $MgSO_4$ and concentrated under vacuum.

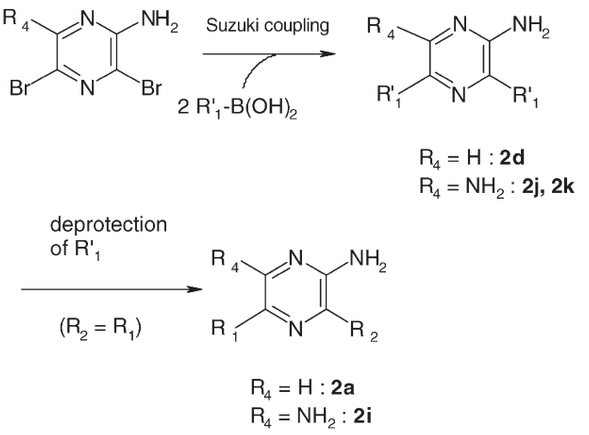
Cyclization reaction. A mixture of 2-amino-1,4-pyrazine (1 equiv.), methyl glyoxal (40 wt% solution in water, 1.5 equiv.), HCl (37% aqueous solution, 3.6 equiv.) in ethanol (10 ml/mmol) was heated at 80°C, under argon atmosphere, for 4 h. After concentration under vacuum, the solid residue was washed successively with ethyl acetate and ether to give imidazolopyrazinone as the hydrated hydrochloride.

Description of the Synthetic Schemes

5-Aryl-2-aminopyrazines **1** (Scheme 2, Table I) resulted from a Suzuki coupling reaction of 5-bromo-2-aminopyrazine with arylboronic acids in the presence of palladium catalyst.[13] The hydroxyl functions present on the aryl moiety (R'^1) were protected as the methyl ethers. Bromination of **1** with *N*-bromosuccinimide gave 3-bromo-5-aryl-2-aminopyrazines, which could suffer a second Suzuki coupling reaction to furnish 3,5-diaryl-2-aminopyrazines **2** bearing two different aryl moieties (R'^1 and R'^2) (Scheme 2). Pure compounds were isolated by



SCHEME 2 Synthesis of unsymmetrically substituted aminopyrazine compounds.



SCHEME 3 Synthesis of symmetrically substituted amino- and diaminopyrazine compounds.

column chromatography on silica gel. The hydroxyl-free derivatives **1** and **2** (with R^1/R^2) were obtained after R'^1/R'^2 deprotection using standard methods, namely the nucleophilic displacement of methyl aryl ether with sodium ethanethiolate (method A),[14] or with aluminium trichloride and ethanethiol (method B).[15]

The symmetrically substituted 3,5-*bis*-aryl-2-aminopyrazines ($R^4 = H$; Scheme 3, Table I) were similarly prepared, from 3,5-dibromo-2-aminopyrazine, by a double Suzuki coupling reaction with arylboronic acids (OH protected) followed by methyl aryl ether deprotection.[6,12] The same two-step strategy could be applied starting from 2,6-diamino-3,5-dibromo-1,4-pyrazine to furnish compounds **2** with $R^4 = NH_2$ (Scheme 3, Table I).[2]

Reference derivatives in which the amine function was missing have been obtained from 2-chloro-1,4-pyrazine and 2,6-dichloro-1,4-pyrazine, respectively: coupling as above with arylboronic acid (OH protected) and deprotection gave compounds **1b–c** and **2b–c** (Table I).

N-Alkylation of 2-aminopyrazines (Scheme 4, Table I) required first the deprotonation with a strong base (lithium hexamethyldisilazanate) and the subsequent reaction of the anion with a halogenoalkane. This reaction was performed on HO-protected derivatives **1** and **2**, with benzylbromide as the alkylating agent. Deprotection of R'^1/R'^2 into R^1/R^2 was conducted as usual.

Lastly, imidazolopyrazinones **3** (Scheme 5, Table II) were readily obtained by condensing 2-aminopyrazines with methylglyoxal in aqueous ethanol and HCl.[11,16] This cyclization reaction was conducted on hydroxyl-free derivatives. Compounds **3** were isolated as the hydrochloride salts.

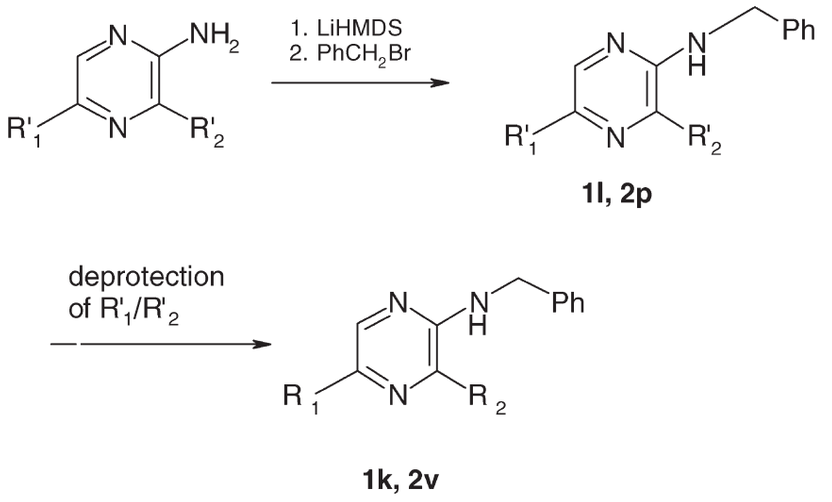
All new derivatives **1**, **2** and **3** (Tables I and II) have been fully characterized by the usual spectroscopic methods and elemental analysis.

2-(4′-methoxyphenyl)-1,4-pyrazine (1c)

The titled compound was prepared from 2-chloro-1,4-pyrazine (0.5 ml, 5.6 mmol) and 4-methoxyphenylboronic acid (894 mg, 5.88 mmol). Yield: 750 mg (72%), white solid; R_F 0.55 (hexane–EtOAc, 3:2); ^{1}H NMR (300 MHz, $CDCl_3$) δ 9.00 (d, 1H, $J = 1.5$ Hz, H-3), 8.60 (dd, 1H, $J = 1.5$ Hz and 2.5 Hz, H-6), 8.46 (d, 1H, $J = 2.5$ Hz, H-5), 8.01 (d, 2H, $J = 8.9$ Hz, aryl), 7.06 (d, 2H, $J = 8.9$ Hz, aryl), 3.9 (s, 3H, OMe); ^{13}C NMR (75 MHz, $CDCl_3$) δ 161.1 (aryl), 152.4 (C-2), 143.9 (C-3), 142.0 (C-5), 141.6 (C-6), 128.8 (aryl), 128.2 (aryl), 114.4 (aryl), 55.3 (OMe); MS (EI, 70 eV) $m/z =$ 186 ($M^{+\bullet}$); Anal. ($C_{11}H_{10}N_2O$) C, H, N.

2-(4′-hydroxyphenyl)-1,4-pyrazine (1b)

Deprotection (method A) of **1c** (320 mg, 1.72 mmol) gave **1b** (296 mg, 65% yield) as a white solid: R_F 0.35 (hexane–EtOAc, 3:2); ^{1}H NMR (200 MHz, DMSO-*d*6) δ 9.92 (s, 1H, OH), 9.12 (d, 1H, $J =$ 1.5 Hz, H-3), 8.60 (dd, 1H, $J = 1.5$ and 2.5 Hz, H-6), 8.47 (d, 1H, $J = 2.5$ Hz, H-5), 7.98 (d, 2H, $J = 8.7$ Hz, aryl), 6.90 (d, 2H, $J = 8.7$ Hz, aryl); ^{13}C NMR (75 MHz, DMSO-*d*6) δ 159.3 (aryl), 151.6 (C-2), 143.9 (C-3), 142.1 (C-5), 141.1 (C-6), 128.2 (aryl), 126.7 (aryl), 115.8 (aryl); MS (EI, 70 eV) $m/z = 172$ ($M^{+\bullet}$); Anal. ($C_{10}H_8N_2O$) C, H, N.



SCHEME 4 *N*-alkylation of aminopyrazine compounds.

2-Amino-5-(2′-methoxyphenyl)-1,4-pyrazine (1′f)

The titled compound was prepared from 2-amino-5-bromo-1,4-pyrazine (495 mg, 2.84 mmol) and 2-methoxyphenylboronic acid (500 mg, 1.1 equiv.). Yield: 517 mg (90.5%), orange solid; mp 98–99°C; R_F 0.10 (cyclohexane–EtOAc, 5:3); ^{1}H NMR (200 MHz, $CDCl_3$): δ 8.53 (d, 1H, $J = 1.4$ Hz, H-3), 8.05 (d, 1H, $J = 1.4$ Hz, H-6), 7.73 (dd, 1H, $J = 7.6$ and 1.7 Hz, aryl), 7.29 (dt, 1H, $J = 8.1$ and 1.7 Hz, aryl), 7.04 (dt, 1H, $J = 7.5$ and 1.7 Hz, aryl), 6.96 (t, 1H, $J = 7.5$ Hz, aryl), 5.40 (s, 2H, NH_2), 3.80 (s, 3H, OMe); ^{13}C NMR (50 MHz, $CDCl_3$): δ 156.6 (aryl), 152.9 (C-2), 144.9 (C-5), 142.3 (C-6), 131.9 (C-3), 130.1 (aryl), 129.4 (aryl), 123.6 (aryl), 121.0 (aryl), 111.3 (aryl), 55.4 (OMe); MS(EI, 70 eV) $m/z = 201$ ($M^{+\bullet}$); Anal. ($C_{11}H_{11}N_3O$) C, H, N.

2-Amino-5-(2′-hydroxyphenyl)-1,4-pyrazine (1f)

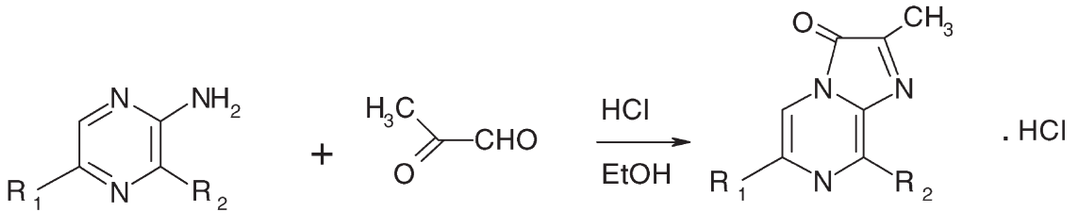
Deprotection (method A) of the 2′-methoxy-precursor (497 mg, 2.47 mmol) gave **1f** (222 mg, 48% yield) as a brown solid: mp 184–186°C (recrystallization from chloroform); ^{1}H NMR (300 MHz, DMSO-*d*6): δ 11.9 (s, 1H, OH), 8.74 (s, 1H, H-3), 7.88 (s, 1H, H-6), 7.83 (m, 1H, aryl), 7.20 (m, 1H, aryl), 6.90 (m, 2H, aryl), 6.67 (s, 2H, NH_2); ^{13}C NMR (75 MHz, DMSO-*d*6): δ 156.20 (aryl), 154.4 (C-2), 140.5 (C-6), 138.9 (C-5), 129.1 (C-3), 128.3 (aryl), 127.9 (aryl), 119.8 (aryl), 119.1 (aryl), 117,12 (aryl); MS (EI, 70 eV) $m/z = 187$ ($M^{+\bullet}$); Anal. ($C_{10}H_9N_3O$) C, H, N.

2-Amino-5-(3′-methoxyphenyl)-1,4-pyrazine (1′g)

The titled compound was prepared from 2-amino-5-bromo-1,4-pyrazine (1.041 g, 5.98 mmol) and 3-methoxy-phenylboronic acid (1 g, 1.1 equiv.). Yield: 807 mg (67%), rose solid; mp 116–117°C; R_F 0.11 (cyclohexane–EtOAc, 5:3); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.44 (d, 1H, $J = 1.4$ Hz, H-3), 8.04 (d, 1H, $J = 1.4$ Hz, H-6), 7.45 (m, 2H, aryl), 7.35 (t, 1H, $J = 7.9$ Hz, aryl), 6.91 (ddd, 1H, $J = 1.2$, 2.4 and 8.0 Hz, aryl), 5.79 (s, 2H, NH_2), 3.87 (s, 3H, OMe); ^{13}C NMR (75 MHz, $CDCl_3$): δ 160.3 (aryl), 153.5 (C-2), 142.7 (C-5), 139.4 (C-6), 138.6 (aryl), 131.7 (C-3), 130.0 (aryl), 118.1 (aryl), 114.3 (aryl), 111.0 (aryl), 55.5 (OMe); MS (EI, 70 eV) $m/z = 201$ ($M^{+\bullet}$); Anal. ($C_{11}H_{11}N_3O$) C, H, N.

2-Amino-5-(3′-hydroxyphenyl)-1,4-pyrazine (1g)

Deprotection (method A) of the 3′-methoxy-precursor (780 mg, 3.88 mmol) gave 1 g (372 mg, 51% yield) as a yellow solid: mp 185–188°C (recrystallization from chloroform); ^{1}H NMR (300 MHz, DMSO-*d*6): δ 11.02 (s, 1H, OH), 9.65 (s, 1H, H-3), 8.41 (s, 1H, H-6), 7.48 (s, 2H, NH_2), 7.25 (m, 2H, aryl), 6.80 (m, 2H, aryl); ^{13}C NMR (75 MHz, DMSO-*d*6): δ 157.8 (aryl),



SCHEME 5 Cyclization reaction of aminopyrazine derivatives into imidazolopyrazinone compounds.

TABLE II Imidazolopyrazinone derivatives

Compound	R^1	R^2	R^3
CLZn	Ph*p*OH	CH_2Ph	CH_2Ph*p*OH
CLZm	Ph*p*OH	CH_2Ph	CH_3
3a	Ph*p*OH	H	CH_3
3b	Ph*p*OCH_3	H	CH_3
3c	Ph	H	CH_3
3d	H	H	CH_3
3e	Ph*o*OH	H	CH_3
3f	Ph*m*OH	H	CH_3
3g	Ph-3,4-diOH	H	CH_3
3h	Ph	Ph*p*OH	CH_3
3i	Ph*p*OH	Ph	CH_3
3j	Ph*p*OH	Ph*p*OH	CH_3
3k	Ph*p*OCH_3	Ph*p*OCH_3	CH_3
3l	Ph-3,4-diOH	Ph*p*OH	CH_3

154.9 (C-2), 139.6 (C-5), 138.8 (C-6), 138.5 (aryl), 131.4 (C-3), 129.7 (aryl), 115.5 (aryl), 115.0 (aryl), 111.6 (aryl); MS (EI, 70 eV) $m/z = 187$ ($M^{+\bullet}$); Anal. ($C_{10}H_9N_3O$) C, H, N; C: calcd, 64.16; found, 64.70.

2,6-*bis*(4′-methoxyphenyl)-1,4-pyrazine (2c)

The titled compound was prepared from 2,6-dichloro-1,4-pyrazine (600 mg, 4.03 mmol) and 4-methoxyphenyl-boronic acid (1.28 g, 8.46 mmol). Yield: 759 mg (64%), white solid; R_F 0.25 (hexane–EtOAc, 3:1); ^{1}H NMR (300 MHz, DMSO-*d*6) δ 9.05 (s, 2H, H-3 + H-5), 8.19 (d, 4H, $J = 8.7$ Hz, aryl), 7.09 (d, 4H, $J = 8.7$ Hz, aryl), 3.82 (s, 6H, OMe); ^{13}C NMR (75 MHz, DMSO-*d*6) δ 160.8 (aryl), 149.9 (C-2, C-6), 138.6 (C-3, C-5), 128.4 (aryl), 128.2 (aryl), 114.4 (aryl), 55.3 (OMe); MS (EI, 70 eV) $m/z = 292$ ($M^{+\bullet}$); Anal. ($C_{18}H_{16}N_2O_2$), C, H, N.

2,6-*bis*(4′-hydroxyphenyl)-1,4-pyrazine (2b)

Deprotection (method A) of **2c** (397 mg, 1.36 mmol) gave **2b** (302 mg, 84% yield) as a white solid; ^{1}H NMR (300 MHz, DMSO-*d*6) δ 9.91 (s, 2H, OH), 8.97 (s, 2H, H-3 + H-5), 8.09 (d, 4H, $J = 7.1$ Hz, aryl), 6.92 (d, 4H, $J = 7.1$ Hz, aryl); ^{13}C NMR (75 MHz, DMSO-*d*6) δ 159.3 (aryl), 150.2 (C-2, C-6), 138.0 (C-3, C-5), 128.3 (aryl), 126.9 (aryl), 115.8 (aryl); MS (EI, 70 eV) $m/z = 264$ ($M^{+\bullet}$); Anal. ($C_{16}H_{12}N_2O_2$) C, H, N; C: calcd, 72.72; found, 71.95.

2-Amino-3-(4′-methoxyphenyl)-5-phenyl-1,4-pyrazine (2f)

The titled compound was prepared from 2-amino-3-bromo-5-phenyl-1,4-pyrazine (2.06 g, 8.24 mmol) and 4-methoxyphenylboronic acid (1.31 g, 1.1 equiv.). Yield: 1.926 g (84%), yellow solid; mp 148–149°C; R_F 0.32 (cyclohexane–EtOAc, 5:3); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.42 (s, 1H, H-6), 7.97 (d, 2H, $J = 7$ Hz, aryl 1), 7.80 (d, 2H, $J = 8.8$ Hz, aryl 2), 7.40 (m, 3H, aryl 1), 7.04 (d, 2H, $J = 8.8$ Hz, aryl 2), 4.87 (s, 2H, NH_2), 3.88 (s, 3H, OMe); ^{13}C NMR (75 MHz, $CDCl_3$): δ 160.2 (aryl 2), 150.9 (C-2), 142.8 (C-5), 139.5 (C-6), 137.1 (C-3, aryl 1), 129.8 (aryl 2), 129.6 (aryl 1), 128.7 (aryl 2), 128.0 (aryl 1), 125.7 (aryl 1), 114.3 (aryl 2), 55.4 (OMe); MS (EI, 70 eV) $m/z = 277$ ($M^{+\bullet}$); Anal. ($C_{17}H_{15}N_3O$) C, H, N; C: calcd, 73.63; found, 73.03.

2-Amino-3-(4′-hydroxyphenyl)-5-phenyl-1,4-pyrazine (2e)

Deprotection (method A) of **2f** (1.929 g, 6.92 mmol) gave **2e** (1.24 g, 68% yield) as a yellow solid: mp 222–223°C; ^{1}H NMR (300 MHz, acetone-*d*6): δ 8.70 (s, 1H, OH), 8.49 (s, 1H, H6), 8.04 (d, 2H, $J = 7.1$ Hz, aryl 1), 7.76 (d, 2H, $J = 8.7$ Hz, aryl 2), 7.40 (m, 3H, aryl 1), 7.00 (d, 2H, $J = 8.7$ Hz, aryl 2), 5.70 (s, 2H, NH_2); ^{13}C NMR (75 MHz, acetone-*d*6): δ 158.8 (aryl 2), 152.8 (C-2), 142.1 (C-5), 140.0 (C-3), 138.5 (C-6), 137.9 (aryl 1), 130.1 (aryl 2), 130.7 (aryl 1), 129.5 (aryl 2), 128.5 (aryl 1), 126.1 (aryl 1), 116.4 (aryl 2); MS (EI, 70 eV) $m/z = 263$ ($M^{+\bullet}$); Anal. ($C_{16}H_{13}N_3O$) C, H, N; C: calcd, 72.99; found, 72.45.

2-Amino-3-phenyl-5-(4′-methoxyphenyl)-1,4-pyrazine (2h)

The titled compound was prepared from 2-amino-3-bromo-5-(4′-methoxyphenyl)-1,4-pyrazine (2.26 g, 8.06 mmol) and phenylboronic acid (1.08 g, 1.1 equiv.). Yield: 1.95 g (87%), yellow solid; mp 124–125°C; R_F 0.31 (cyclohexane–EtOAc, 5:3); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.40 (s, 1H, H-6), 7.92 (d, 2H, $J = 8.9$ Hz, aryl 1), 7.83 (d, 2H, $J = 8.1$ Hz, aryl 2), 7.50 (m, 3H, aryl 2), 6.98 (d, 2H, $J = 8.9$ Hz, aryl 1), 4.80 (s, 2H, NH_2), 3.86 (s, 3H, OMe); ^{13}C NMR (75 MHz, $CDCl_3$): δ 159.8 (aryl 1), 150.4 (C-2), 142.9 (C-5), 139.4 (C-6), 137.5 (C-3), 137.1 (aryl 2), 129.8 (aryl 1), 128.9 (aryl 2), 128.3 (aryl 1), 126.9 (aryl 2), 114.1 (aryl 1), 55.3 (OMe); MS (EI, 70 eV) $m/z = 277$ ($M^{+\bullet}$); Anal. ($C_{17}H_{15}N_3O$) C, H, N.

2-Amino-3-phenyl-5-(4′-hydroxyphenyl)-1,4-pyrazine (2g)

Deprotection (method A) of **2h** (1.92 g, 6.92 mmol) gave **2g** (1.25 g, 69% yield) as a yellow solid: mp 200–201°C; ^{1}H NMR (200 MHz, acetone-*d*6): δ 8.49 (s, 1H, OH), 8.46 (s, 1H, H-6), 7.90 (m, 4H, aryl 1 + aryl 2), 7.50 (m, 3H, aryl 2), 6.94 (d, 2H, $J =$ 8.9 Hz, aryl 1), 5.60 (s, 2H, NH_2); ^{13}C NMR (50 MHz, acetone-*d*6): δ 158.6 (aryl 1), 152.3 (C-2), 142.9 (C-5), 139.4 (C-6), 139.2 (C-3), 137.9 (aryl 2), 130.1 (aryl 1), 129.6 (aryl 2), 129.5 (aryl 2), 129.3 (aryl 1), 127.7 (aryl 2), 116.5 (aryl 1); MS (EI, 70 eV) $m/z = 263$ ($M^{+\bullet}$); Anal. ($C_{16}H_{13}N_3O$) C, H, N; C: calcd, 72.99; found, 72.33.

2-Methyl-6-(2′-hydroxyphenyl)-3,7-dihydroimidazolo[1,2-a]pyrazin-3-one (3e)

Reaction of **1f** (435 mg, 2.35 mmol) with methylglyoxal (40 wt% in water, 0.54 ml, 1.5 equiv.) and HCl (37 wt% in water, 0.71 ml, 3.6 equiv.) in ethanol (11 ml) gave **3e** (547 mg, 79% yield) as a pale brown solid: mp 230–232°C (dec.); ^{1}H NMR (300 MHz, DMSO-*d*6): δ 11.5 (s, 1H, OH), 9.40 (s, 1H, H-8), 9.07 (s, 1H, H-5), 7.96 (d, 1H, $J = 6.5$ Hz, aryl), 7.26 (m, 3H, aryl), 2.26 (s, 3H, Me); ^{13}C NMR (75 MHz, DMSO-*d*6): δ 156.6 (aryl), 154.8 (C-8), 133.1 (aryl), 132.1 (C-9), 130.7 (C-6), 130.4 (aryl), 128.0 (aryl), 127.6 (aryl), 119.7 (C-2, C-3), 119.4 (aryl), 117.2 (C-5), 14.4 (Me); MS (FAB) $m/z = 242$ $(M + 1)^+$; Anal. ($C_{13}H_{11}N_3O_2$·HCl·H_2O): calcd C, 52.75; H, 4.73; Cl, 12.00; N, 14.20—Found C, 52.62; H, 5.39; Cl, 11.51; N, 14.82.

2-Methyl-6-(3′-hydroxyphenyl)-3,7-dihydroimidazolo[1,2-a]pyrazin-3-one (3f)

Reaction of **1g** (177 mg, 0.95 mmol) with 40% methylglyoxal (0.22 ml), and 37% HCl (0.28 ml) in ethanol (8 ml) gave **3f** (224 mg, 83% yield) as a brown solid: mp 225–227°C (dec.); ^{1}H NMR (300 MHz, DMSO-*d*6): δ11.3 (s, 1H, OH), 9.91 (s, 1H, H-8), 8.75 (s, 1H, H-5), 7.40 (m, 3H, aryl), 6.92 (d, 1H, $J = 7.5$ Hz, aryl), 2.51 (s, 3H, Me); ^{13}C NMR (75 MHz, DMSO-*d*6): δ 158.0 (aryl), 150.4 (C-8), 135.0 (aryl), 134.2 (C-9), 130.1 (aryl), 127.1 (C-6), 117.0 (aryl), 116.6 (aryl), 115.6 (C-3), 115.3 (C-2), 113.3 (aryl), 111.2 (C-5), 10.4 (Me); MS (FAB) $m/z = 242$ $(M + 1)^+$; Anal. ($C_{13}H_{11}N_3O_2$·HCl·0.5 H_2O) C, H, N.

2-Methyl-6-phenyl-8-(4′-hydroxyphenyl)-3,7-dihydroimidazolo[1,2-a]pyrazin-3-one (3h)

Reaction of **2e** (279 mg, 1.06 mmol) with 40% methylglyoxal (0.25 ml) and 37% HCl (0.32 ml) in ethanol (4 ml) gave **3h** (324 mg, 82% yield) as a yellow solid: mp 99–101°C (dec.); ^{1}H NMR (200 MHz, CD_3OD): δ 8.49 (s, 1H, H-5), 7.97 (m, 2H, aryl 1), 7.89 (d, 2H, $J = 8.8$ Hz, aryl 2), 7.40 (m, 3H, aryl 1), 6.95 (d, 2H, $J = 8.8$ Hz, aryl 2); 2.44 (s, 3H, Me); ^{13}C NMR (50 MHz, CD_3OD); δ 162.7 (aryl 2), 146.2 (C-8), 142.1 (C-9), 139.1 (C-3), 135.6 (aryl 1), 131.1 (aryl 2), 132.2 (aryl 1), 130.2 (aryl 1), 128.0 (aryl 1), 126.8 (aryl 2), 125.1 (C-6), 121.3 (C-2), 117.3 (aryl 2), 110.5 (C-5), 9.7 (Me); MS (FAB) $m/z = 318$ $(M + 1)^{+\bullet}$; Anal. ($C_{19}H_{15}N_3O_2$·HCl·1.2 H_2O) C, HN.

2-Methyl-6-(4′-hydroxyphenyl)-8-phenyl-3,7-dihydroimidazolo[1,2-a]pyrazin-3-one (3i)

Reaction of **2g** (450 mg, 1.71 mmol) with 40 % methylglyoxal (0.4 ml) and 37% HCl (0.51 ml) in ethanol (7 ml) gave **3i** (500 mg, 79% yield) as a yellow solid: mp 179–181°C (dec.); ^{1}H NMR (200 MHz, DMSO-*d*6): δ 8.74 (s, 1H, H-5), 8.15 (m, 2H, aryl 2), 7.98 (d, 2H, $J = 7.9$ Hz, aryl 1), 7.65 (m, 3H, aryl 2), 6.92 (d, 2H, $J = 7.9$ Hz, aryl 1), 2.09 (s, 3H, Me); ^{13}C NMR (50 MHz, DMSO-*d*6): δ 158.8 (aryl 1), 144.1 (C-8), 140.0 (aryl 2), 136.8 (C-9), 133.8 (C-6), 131.0 (aryl 2), 129.1 (aryl 2), 128.8 (aryl 2), 128.3 (aryl 1), 127.8 (aryl 1), 125.5 (C-3), 125.2 (C-2), 115.7 (aryl 1), 109.6 (C-5), 9.8 (Me); MS (FAB) $m/z = 318$ $(M + 1)^{+\bullet}$; Anal. ($C_{19}H_{15}N_3O_2$·HCl·1.2H_2O) C, H, N.

2-Methyl-6,8-*bis*(4′-methoxyphenyl)-3,7-dihydroimidazolo[1,2-a]pyrazin-3-one (3k)

Reaction of **2d** (535 mg, 1.74 mmol) with 40% methylglyoxal (0.4 ml) and 37% HCl (0.52 ml) in ethanol (6 ml) gave **3k** (636 mg, 92% yield) as a yellow solid: ^{1}H NMR (300 MHz, DMSO-*d*6) δ 8.65 (s, 1H, H-5), 8.32 (d, 2H, $J = 8.1$ Hz, aryl 1), 8.11 (d, 2H, $J = 7.9$ Hz, aryl 2), 7.18 (d, 2H, $J = 7.9$ Hz, aryl 2), 7.08 (d, 2H, $J = 8.1$ Hz, aryl 1), 3.88 (s, 3H, OMe), 3.83 (s, 3H, OMe), 2.46 (s, 3H, Me); ^{13}C NMR (75 MHz, DMSO-*d*6) δ 161.5 (aryl 1), 160.2 (aryl 2), 143.7 (C-8, C-9), 139.0 (C-3), 136.9 (C-6), 130.8 (aryl 1), 127.7 (aryl 2), 127.2 (aryl 1), 126.0 (aryl 2), 125.2 (C2), 114.2 (aryl 1 and aryl 2), 108.5 (C-5), 9.94 (Me); MS (FAB^+) $m/z = 362$ $(M + 1)^+$; Anal. ($C_{21}H_{19}N_3O_3$·HCl) C, H, N; C: calcd, 63.39; found, 62.40.

2-(*N*-Benzylamino)-5-(4′-methoxyphenyl)-1,4-pyrazine (1l)

The titled compound was prepared from **1e** (500 mg, 2.48 mmol), LiHMSD (900 mg) and benzylbromide (467 mg). Yield: 303 mg (42%), yellow solid; mp 153–154°C; R_F 0.57 (cyclohexane–EtOAc, 5:3); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.39 (d, 1H, $J = 1.2$ Hz, H-3), 7.92 (d, 1H, $J = 1.2$ Hz, H-6), 7.78 (d, 2H, $J = 8.8$ Hz, aryl), 7.32 (m, 5H, Ph), 6.95 (d, 2H, $J = 8.8$ Hz, aryl), 5.09 (t, 1H, $J = 5.8$ Hz, NH), 4.57 (d, 2H, $J = 5.8$ Hz, CH_2), 3.83 (s, 3H, OMe); ^{13}C NMR (75 MHz, $CDCl_3$): δ 159.8 (aryl), 152.9 (C-2), 141.9 (C-5), 138.6 (C-6, Ph), 130.9 (C-3), 130.1 (aryl), 128.9 (aryl), 127.7 and 126.9 (Ph), 114.4 (aryl), 55.5 (OMe), 46.0 (CH_2); MS (EI, 70 eV) $m/z =$ 291 ($M^{+\bullet}$); Anal. ($C_{18}H_{17}N_3O$·0.15 H_2O) C, H, N.

2-(*N*-Benzylamino)-5-(4′-hydroxyphenyl)-1,4-pyrazine (1k)

Deprotection (Method B) of **1l** (289 mg, 0.99 mmol) gave **1k** (76 mg, 27% yield) as a yellow solid: mp 159–160°C; R_F 0.60 (cyclohexane–EtOAc, 5:2); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.40 (d, 1H, $J = 1.4$ Hz, H-3), 7.94 (d, 1H, $J = 1.4$ Hz, H-6), 7.73 (d, 2H, $J =$ 8.7 Hz, aryl), 7.40 (m, 5H, Ph), 6.87 (d, 2H, $J = 8.7$ Hz, aryl), 5.00 (m, 1H, NH), 4.60 (d, 2H, $J = 5.8$ Hz, CH_2); ^{13}C NMR (75 MHz, $CDCl_3$): δ 156.2 (aryl), 152.6 (C-2), 143.6 (C-5), 138.5 (Ph), 138.0 (C-6), 130.9 (C3), 129.6 (aryl), 129.0 (aryl), 127.8 (Ph), 127.7 (Ph), 127.3 (Ph),

116.0 (aryl), 46.2 (CH_2); MS (EI, 70 eV) $m/z = 277$ ($M^{+\bullet}$); Anal. ($C_{17}H_{15}N_3O$) C, H, N.

2-(*N*-Benzylamino)-3,5-*bis*(4′-methoxyphenyl)-1,4-pyrazine (2p)

The titled compound was prepared from **2d** (500 mg, 1.63 mmol), LiHMDS (592 mg) and benzylbromide (306 mg). Yield: 404 mg (62%), yellow solid; mp 102–103°C; R_F 0.44 (cyclohexane–EtOAc, 5:1); ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.40 (s, 1H, H-6), 7.88 (d, 2H, $J = 8.9$ Hz, aryl 2), 7.71 (d, 2H, $J = 8.8$ Hz, aryl 1), 7.33 (m, 5H, Ph), 7.01 (d, 2H, $J = 8.8$ Hz, aryl 1), 6.96 (d, 2H, $J = 8.9$ Hz, aryl 2), 5.27 (t, 1H, $J = 5.4$ Hz, NH), 4.66 (d, 2H, $J = 5.4$ Hz, CH_2), 3.84 (s, 6H, OMe × 2); ^{13}C NMR (75 MHz, $CDCl_3$): δ 160.3 and 159.7 (aryl 1, aryl 2), 150.5 (C-2), 141.1 (C-5), 140.3 (C3), 139.4 (Ph), 136.4 (C-6), 130.6 (aryl 1), 130.0 (aryl 2), 129.7 (aryl 2), 128.8 (Ph), 127.7 (Ph), 127.4 (Ph), 127.0 (aryl 1), 114.7 (aryl 2), 114.3 (aryl 1), 55.5 (OMe × 2), 45.8 (CH_2); MS (EI, 70 eV) $m/z = 397$ ($M^{+\bullet}$); Anal. ($C_{25}H_{23}N_3O_2$) C, H, N.

2-(*N*-Benzylamino)-3,5-*bis*(4′-hydroxyphenyl)-1,4-pyrazine (2o)

Deprotection (Method B) of **2p** (200 mg, 0.5 mmol) gave **2o** (147 mg, 79% yield) as a yellow solid: mp 90–91°C; R_F 0.53 (cyclohexane–EtOAc, 5:2); ^{1}H NMR (200 MHz, acetone-*d*6): δ 8.47 (s, 1H, OH), 8.23 (s, 1H, OH), 8.15 (s, 1H, H-6), 7.62 (d, 2H, $J = 8.8$ Hz, aryl 2), 7.44 (d, 2H, $J = 8.7$ Hz, aryl 1), 7.05 (m, 5H, Ph), 6.72 (d, 2H, $J = 8.7$ Hz, aryl 1), 6.66 (d, 2H, $J = 8.8$ Hz, aryl 2), 5.27 (t, 1H, $J = 5.9$ Hz, NH), 4.46 (d, 2H, $J = 5.9$ Hz, CH_2); ^{13}C NMR (50 MHz, acetone-*d*6): δ 159.00 and 158.4 (aryl 1, aryl 2), 151.5 (C-2), 141.6 (C-5), 140.3 (C-3), 141.0 (Ph), 136.5 (C-6), 131.0 (aryl 2), 130.3 (aryl 1), 129.9 (aryl 2), 129.2 (Ph), 128.4 (Ph), 127.5 (Ph, aryl 1), 116.6 (aryl 1), 116.4 (aryl 2), 45.6 (CH_2); MS (EI, 70 eV) $m/z = 369$ ($M^{+\bullet}$); Anal. ($C_{23}H_{19}N_3O_2 \cdot 0.5\ H_2O$) C, H.

Measurement of Lipophilicity

The relative lipophilicity of some compounds used in this study was estimated by their partition coefficients in an octanol/water mixture. In brief, compounds (50 μM) in 100 mM phosphate buffered saline, pH 7.4, were mixed vigorously with an equal volume of *n*-octanol. The mixture was centrifuged, and the fluorescence emission in the aqueous layer was measured using in a fluorimeter (Kontron Instruments) and compared to that of the starting solution. Excitation–emission wavelengths were as follows: CLM (351 nm/441 nm), compound **1a** (355 nm/443 nm), compound **2a** (364 nm/460 nm), compound **2f** (364 nm/464 nm), compound **1k** (361 nm/452 nm), compound **2o** (367 nm/469 nm). The partition coefficient$_{(\text{octanol/water})}$ ($\log P$) was calculated according to the relationship where P is defined as

$$P = \frac{A_0 - A_x}{A_x} = \frac{C_{\text{octanol}}}{C_{\text{water}}}$$

where A_0 is the initial fluorescence value of the compound in the aqueous layer before mixing with octanol layer, and A_x is the final fluorescence level in the aqueous layer after mixing with octanol. C_{octanol} and C_{aq} are the concentrations of the compounds in octanol and aqueous layer, respectively. If a compound is equally distributed in octanol and aqueous layers after mixing, P is equal to 1 and $\log P$ is 0. A compound with $\log P$ of 1 indicates that it is 10 times more soluble in octanol than in the aqueous buffer.

Lipid Peroxidation Assay

A micellar solution of linoleic acid (0.16 mM) in 50 mM phosphate buffer, pH 7.4, is incubated at 37°C with 2 mM AAPH (2, 2′-azo-*bis*(2-amidinopropane)-dihydrochloride) as the free-radical generator. The production of conjugated dienes by the peroxidation of linoleate is monitored continuously at 234 nm using a wavelength tuneable microplate spectrophotometer reader (SpectraMAX 190, Molecular Devices). Antioxidant efficiency of the tested compound was evaluated by the lag phase duration until onset of peroxidation and by the inhibition of the propagation rate calculated with the following equation:

$$\text{Inh}\,(\%) = 100 - \frac{R_{\text{inh}}}{100R_0}$$

where R_{inh}/R_0 is the ratio of the rate of oxidation inhibited by tested compound (R_{inh}) to that of the uninhibited oxidation (R_0).

In parallel to the measurement of the production of conjugated dienes, the concentration of some IMPZs (at 10 μM) was monitored at a wavelength (420 nm) specific to the fused imidazolo-pyrazinone ring and not to their oxidation products.

Statistical Analysis

ANOVA and Tukey tests were used for comparing the efficiency of tested compounds among groups.

RESULTS

All compounds were evaluated for their ability to protect linoleic acid micelles from oxidation initiated by the water-soluble free radical initiator 2,2′-azo-*bis*(2-amidinopropane) dihydrochloride (AAPH). The kinetics of conjugated dienes production during AAPH-catalyzed oxidation are

presented in Fig. 1. The addition of the azo initiator to the micellar solution caused a constant production of conjugated dienes until reaching a plateau (Fig. 1).

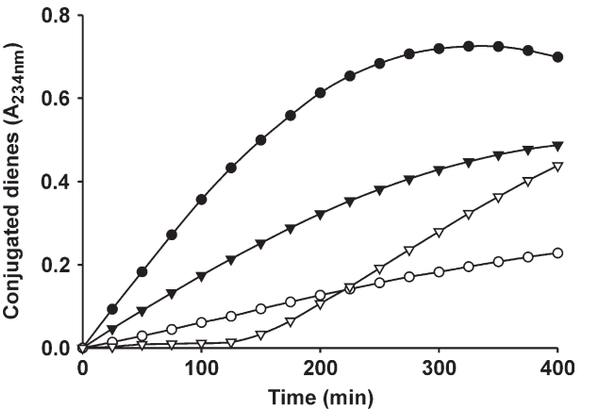


FIGURE 1 Effect of CLM, compound **1a** and CLZm (10 μM) on AAPH-induced lipid peroxidation assessed by the rate of conjugated dienes formation. Control (●), CLM (○), compounds **1a** (▼) and CLZm (▽). Values are means ± S.D. of triplicate samples. Variation was less than 1% and error bars are smaller than the symbol size.

CLM and Derivatives

CLM (10 μM) added to the micellar solution inhibited the AAPH-induced linoleate peroxidation as evidenced by a decreased propagation rate of oxidation. The influence of benzyl (R^2), amino (R^5) and *p*-hydroxyphenyl (R^1) groups on the antioxidant activity of CLM was investigated. Replacement of the C_3-benzyl group (R^2) in CLM by *p*-hydroxyphenyl or phenyl groups as in compounds **2a** and **2g** respectively, did not significantly affect the antioxidant activity (Table III). In contrast, compound **1a** where the C_3-benzyl group was replaced by a hydrogen atom was not as effective as CLM ($p < 0.001$), reaching 17 and 54% of inhibition of the oxidation rate at 5 and 10 μM, whereas inhibitions reached 61 and 79% for CLM. The partition coefficients$_{(octanol/water)}$ (log *P*) of CLM, compounds **2a**, **2g** and **1a** were 1.09, 1.95, 1.85 and 0.13, respectively (Table I). These results indicate that a high lipophilicity of AMP molecules is not crucial for the antioxidant activity, but it improves the inhibitory action on lipid micelles.

Derivatives lacking the 2-amino group such as compounds **1b** and **2b** showed reduced activities compared to compounds **1a** and **2a**, respectively, ($p < 0.001$). On the other hand, compounds **1d** and

TABLE III Inhibition of AAPH-induced lipid peroxidation by 1,4-pyrazine derivatives and their corresponding imidazolopyrazinones

	Concentration			
	5 μM		10 μM	
	% Inhibition oxidation rate†	Lag phase (min)	% Inhibition oxidation rate†	Lag phase (min)
CLM	61.50 ± 1.11***	nd	79.20 ± 0.52***	nd
1a	17.02 ± 1.64	nd	53.77 ± 3.48***	nd
1b	7.32 ± 1.36	nd	17.76 ± 5.60	nd
1c	0.00 ± 2.07	nd	6.01 ± 2.06	nd
1d	0.60 ± 0.02	nd	1.81 ± 0.81	nd
1e	1.04 ± 0.07	nd	3.43 ± 2.3	nd
1f	6.50 ± 1.66	nd	12.35 ± 0.52*	nd
1g	5.06 ± 1.18	nd	6.27 ± 0.52	nd
2a	65.62 ± 1.06***	nd	75.35 ± 0.69***	nd
2b	38.83 ± 1.65***	nd	51.54 ± 2.21***	nd
2c	1.08 ± 0.75	nd	3.19 ± 3.05	nd
2d	0.02 ± 0.14	nd	0.00 ± 0.02	nd
2e	33.40 ± 0.40***	nd	51.93 ± 2.77***	nd
2f	0.04 ± 0.00	nd	1.81 ± 1.75	nd
2g	65.50 ± 1.30***	nd	75.32 ± 1.47***	nd
2h	0.00 ± 0.47	nd	0.00 ± 2.88	nd
CLZm	45.65 ± 1.47***	102.15 ± 5.66	50.14 ± 0.82***	122.58 ± 1.34
3a	30.44 ± 1.51**	113.31 ± 3.73	29.88 ± 1.20***	179.63 ± 2.34***
3b	3.12 ± 1.53	120.94 ± 1.94	0.00 ± 2.01	164.20 ± 8.07***
3c	2.04 ± 4.89	128.14 ± 3.64	0.00 ± 1.15	163.43 ± 0.44***
3d	0.00 ± 0.52	133.54 ± 3.32	0.00 ± 1.19	222.76 ± 1.02***
3e	25.47 ± 14.0*	138.75 ± 5.86	8.00 ± 1.16	197.85 ± 3.57***
3f	8.30 ± 1.37	102.66 ± 3.71	0.00 ± 0.63	141.68 ± 3.97***
3h	28.53 ± 0.69**	119.01 ± 0.54	32.65 ± 2.98***	185.16 ± 5.27***
3i	53.76 ± 1.05***	138.25 ± 3.00	60.58 ± 0.68***	189.91 ± 4.38***
3j	63.39 ± 8.38***	136.95 ± 0.67	72.92 ± 2.18***	198.01 ± 11.5***
3k	11.79 ± 1.74	147.74 ± 3.42	2.19 ± 2.91	200.87 ± 7.82***

† Percentage of inhibition of the oxidation rate (after the lag phase) estimated by the production rate of conjugated dienes in linoleate micelles incubated with AAPH at 37°C. The inhibition of the oxidation rate by tested compounds was compared to that induced by AAPH alone which was 4.25 ± 0.05 mUAbs$_{234nm}$/min. Values represent means ± S.D. of triplicate experiments. Statistical comparisons were made with the uninhibited peroxidation rate. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. nd indicates no lag-phase detected.

2c lacking the hydroxyl group were totally devoid of antioxidant activity. As expected, similar results were obtained for the compounds where the *p*-hydroxyphenyl group was replaced with a *p*-methoxyphenyl group as in compounds **1c**, **1e**, **2d**, **2f** and **2h**. This indicates that both the 2-amino and the 5-*p*-hydroxyphenyl groups are essential features required for the chain-breaking activity of CLM. Nevertheless, the *p*-hydroxyphenyl group is more important than the 2-amino for this antioxidant activity (**1b** vs. **1d** and **1e**). These results are consistent with the hypothesis that the antioxidant activity of CLM and 2-amino-5-(*p*-hydroxyphenyl)-1,4-pyrazines resides in the ability of the hydroxyl group to donate a hydrogen, resulting in a phenoxyl radical stabilized by conjugative interaction with 2-amino-1,4-pyrazine representing a semi-quinonic system. Accordingly the electronic resonance between the 2-amino and 5-phenoxyl radical should be maximal when the hydroxyl group is located in para position on the phenyl substituent, and so the antioxidant activity. Indeed, compounds having hydroxyl groups in ortho and meta positions (compounds **1f** and **1g**) were significantly ($p < 0.001$) much less active than the 2-amino-5-(*p*-hydroxyphenyl)-1,4-pyrazine (compound **1a**). This is confirmed by derivatives varying by the position (C_3 vs. C_5) of the phenol substituent: as far as the 3,5-diaryl-substituted compounds are concerned, the *p*-hydroxyphenyl group conferred a higher activity when located in para position with respect to the amino group as in compound **2g** rather than in ortho (compound **2e**).

CLZm Derivatives

All tested IMPZs (Fig. 1 and Table III) delayed the onset of lipid peroxidation with significant differences appearing at 10 μM. The lag time of linoleate peroxidation in the presence of 10 μM CLZm was 122.6 ± 1.4 min. This was significantly extended when the C_8-benzyl group was replaced by a hydrogen atom (compound **3a**), a *p*-hydroxyphenyl group (compound **3j**) or a phenyl group (compound **3i**) ($p < 0.001$). Structural modifications of the C_6-*p*-hydroxyphenyl group of compound **3a**, giving compounds **3b**, **3c**, **3e** and **3f**, did not increase further the lag time characterizing compound **3a**. Similar results were obtained for C_6, C_8-disubstituted IMPZs, i.e., compounds **3h**, **3i**, **3j** and **3k**. Finally, the lag time for compound **3d** lacking C_6 or C_8 substituents was prolonged in comparison to that induced by CLZm. All these data suggest that C_6 and C_8 substituents are not necessary for delaying the reaction. However, these substituents appeared to influence the rate at which the oxidation resumes after the lag phase. *p*-Hydroxyphenyl substituted IMPZs (compounds **3a**, **3i** and **3j**) reduced significantly the oxidation rate observed after the lag phase compared to AAPH alone ($p < 0.001$). By contrast, IMPZs whose *p*-hydroxyphenyl substituents were replaced with an hydrogen atom (compound **3d**), a phenyl (compound **3c**) or a methoxyphenyl group (compounds **3b** and **3k**), had no effect on the rate of linoleate oxidation. The reduction of the oxidation rate was maximal when the hydroxyl group is located at para position on the C_6-phenyl substituent (compound **3a**) instead of ortho or meta positions (compounds **3e** and **3f**) ($p < 0.001$). In addition, compound **3i**, which *p*-hydroxyphenyl group is located at C_6 was significantly more active than compound **3h** bearing a phenyl group at C_6 and a hydroxyphenyl group at C_8 ($p < 0.05$ and $p < 0.001$ at 5 and 10 μM, respectively). Therefore, our data indicated that only phenol-substituted IMPZs efficiently reduce the oxidation rate. As illustrated in Fig. 2 for compounds **3d** and **3a**, IMPZs are consumed at a constant rate during the inhibition period (lag time).The inhibition period ended when IMPZs were almost fully oxidized. This suggests that IMPZs are not directly responsible for the inhibition of the oxidation rate. In contrast, it is likely that the corresponding AMPs generated by the reaction of IMPZs with ROS produced during lipid peroxidation most probably account for this inhibition.

Design of New CLM Derivatives with Improved Antioxidant Properties

In the second part of this work, we examined the possibility of improving the antioxidant activities of AMPs. First, the antioxidant activity of compounds **1k** and **2o**, resulting from the introduction of a benzyl substituent on the 2-amino group of **1a** and **2a**, respectively (Table IV) was tested. This was performed at 2.5 and 5 μM in order to better discriminate differences in activity. These structural modifications markedly affected the antioxidant activity as the inhibition reached 60 and 78% for compounds **1k** and **2o** (2.5 μM) compared respectively to 6 and 43% for compounds **1a** and **2a**, at 2.5 μM ($p < 0.001$). This increased activity is most probably due to differences in lipophilicity. Indeed, the increasing ranking order of partition coefficients is: compound **1a**(0.13) < compound **1k**(1.16) < compound **2a**(1.95) < compound **2o** (>2). The increased basicity of the amine induced by the benzyl group may also potentiate the electron releasing effect of the amino group and so the stabilisation of the phenoxyl radical. Again, the corresponding (*p*-methoxyphenyl)-1,4-pyrazines (i.e., compounds **1l** and **2p**) had no effect on lipid peroxidation.

As the presence of both amine and hydroxyl functions is essential to the antioxidant activity of CLM, we tested whether the introduction of either

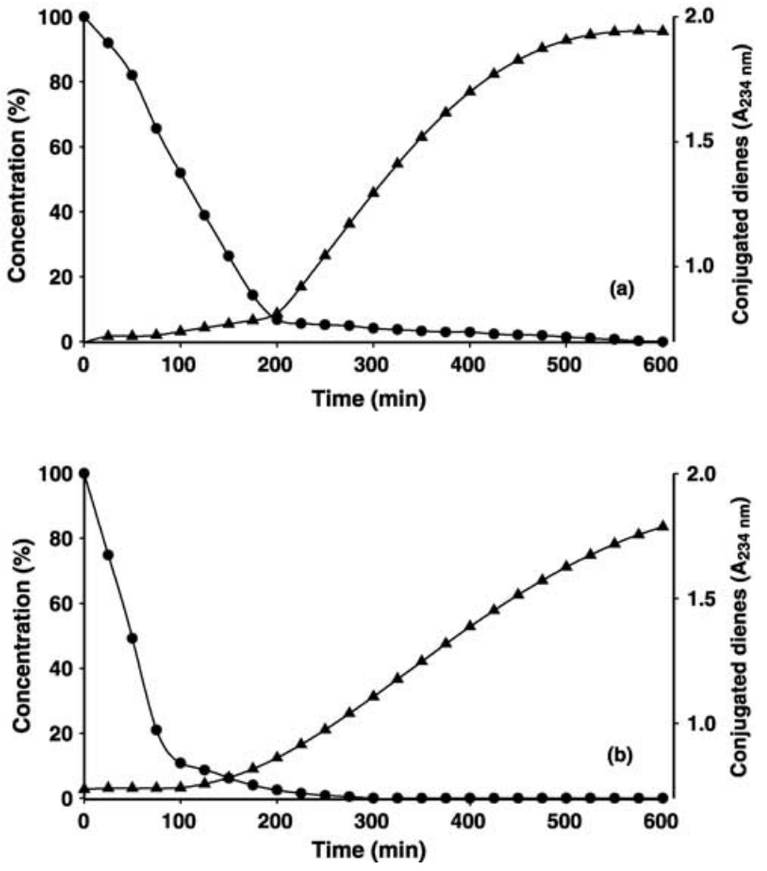


FIGURE 2 Consumption of imidazolopyrazinones and their inhibitory effect on lipid peroxidation. A. Compound **3d**; B. compound **3a**. The concentrations of compounds **3a** and **3d** were monitored at 420 nm where oxidation products show no absorption and the levels of conjugated dienes were followed at 234 nm. (•), concentration; (▲) conjugated dienes. Each curve is an average of triplicate experiments.

a second amine at the C_6 position of the pyrazine nucleus or a second hydroxyl group on the *p*-hydroxyphenyl group, resulting in a catechol function, could improve the antioxidant activity. Since lipid peroxidation never started when these compounds were applied at 10 μM, tests were carried out at 2.5 and 5 μM (Table IV).

Previous studies have shown that 2,6-diamino-3,5-diaryl substituted AMP behaved like IMPZ derivatives as they delayed the onset of AAPH-induced lipid peroxidation.[12] After this delay, the oxidation proceeds at a similar rate as that in the absence of antioxidant. The occurrence of the two amino groups at C_2 and C_6 accounted in part for the antioxidant activity of compound **2j** as the removal of the *p*-hydroxyphenyl groups giving compound **2i** did not suppress the lag time observed with compound **2j**. However, C_3 and C_5 (*p*-hydroxyphenyl) groups also play a role as their replacement by methoxyphenyl (compound **2k**) or phenyl groups (compound **2l**) decreased significantly the lag time compared to compound **2j**. Compound **2j** was more active than vitamin E and Trolox C, a hydrosoluble analog of vitamin E, and the polyphenolic compound epigallocatechin gallate (EGCG) extracted from green tea leaves.[17,18]

Replacement of the *p*-hydroxyphenyl group with a catechol moiety at C_5 position of the pyrazine nucleus of 5-mono- and 3,5-diaryl substituted AMP yielded compounds **1h** and **2n**. Both compounds induced a lag phase. After this lag time, the oxidation proceeded at a rate similar (at 2.5 μM) or lower (at 5 μM) than that observed for their respective hydroxyphenyl-substituted derivatives, i.e., compounds **1a** and **2a** (Table IV). The introduction of a *p*-hydroxyphenyl function at C_3 position of the pyrazine nucleus of compound *1h* resulting in compound **2n** did not increase significantly the antioxidant activity. The position of the catechol group (C_3, compound **2m**; C_5, compound **2n**) on the pyrazine ring, was not crucial for high antioxidant activity. However, the relative positions of both hydroxyl groups are important for the full antioxidant activity of the catechol-substituted compounds. Indeed, compound **1j** whose hydroxyl functions are located at 2- and 4-positions of the C_5-benzyl moiety showed a reduced lag time compared to that produced by compound **1h**.

TABLE IV Inhibitory effects of new 1,4-aminopyrazine derivatives and their corresponding imidazopyrazinones on lipid peroxidation

	Concentration			
	2.5 μM		5 μM	
	% Inhibition of oxidation rate[†]	Lag phase (min)	% Inhibition of oxidation rate[†]	Lag phase (min)
CLM	39.26 ± 0.31***	nd	61.50 ± 1.11***	nd
1a	5.89 ± 1.05	nd	17.02 ± 1.64	nd
1h	22.90 ± 5.80	270.50 ± 3.13	26.08 ± 0.83*	398.74 ± 0.97
1i	0.13 ± 0.69	nd	0.02 ± 1.00	nd
1j	10.06 ± 2.54	116.25 ± 17.38	22.34 ± 1.55	163.09 ± 2.14
1k	59.94 ± 3.57***	nd	71.51 ± 1.54***	nd
1l	2.18 ± 0.85	nd	4.53 ± 1.79	nd
2a	42.72 ± 1.22***	nd	65.62 ± 1.08***	nd
2j	26.27 ± 2.77*	255.08 ± 16.29*	16.39 ± 2.93*	420.08 ± 17.46
2k	0.00 ± 0.22	124.37 ± 2.47	5.38 ± 1.28	196.52 ± 7.92
2l	0.01 ± 2.50	112.35 ± 1.74	24.31 ± 2.64*	150.44 ± 8.80
2m	33.99 ± 5.35***	250.55 ± 6.49	48.43 ± 4.34***	367.47 ± 12.72
2n	10.37 ± 2.10	257.35 ± 2.20	39.55 ± 1.82***	404.18 ± 2.81
2o	78.65 ± 2.24***	nd	81.36 ± 0.56***	nd
2p	0.21 ± 0.70	nd	0.19 ± 0.76	nd
3a	11.10 ± 1.24	80.15 ± 0.45	30.44 ± 1.51**	113.31 ± 3.73
3g	24.95 ± 17.46*	260.81 ± 18.66	21.10 ± 0.63	360.91 ± 3.89
3j	41.03 ± 1.49***	171.84 ± 9.96	63.39 ± 8.38***	136.95 ± 0.67
3l	17.80 ± 1.71	239.47 ± 3.07	35.36 ± 4.32***	365.91 ± 17.46
Catéchol	51.62 ± 1.40***	nd	72.39 ± 0.40***	nd
Vitamin E	0.59 ± 1.34	170.99 ± 17.34	4.78 ± 3.01	252.68 ± 13.23
Trolox	0.46 ± 3.26	102.01 ± 0.35	0.00 ± 1.04	120.17 ± 4.05
EGCG	6.57 ± 1.97	214.66 ± 7.61	21.21 ± 3.13	365.91 ± 17.46

[†] Percentage of inhibition of the oxidation rate (after the lag phase) estimated by the production rate of conjugated dienes in linoleate micelles incubated with AAPH at 37°C. The inhibition of the oxidation rate by tested compounds was compared to that induced by AAPH alone which was 4.25 ± 0.05 $mUAbs_{234\,nm}$/min. Values represent means ± S.D. of triplicate experiments. Statistical comparisons were made with the uninhibited peroxidation rate. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. nd indicates no lag-phase detected.

The presence of 3,4-dihydroxyl functions on the C_5-phenyl group seemed to be essential for the high antioxidant activity of compound **1h** since the methylation of both hydroxyl groups, (compound **1i**), suppressed the antioxidant activity. However, the catechol function by itself did not account for the entire activity of catechol-substituted AMP derivatives. Indeed, catechol itself (1,2-dihydroxybenzene) alone gave no lag time and only reduced the rate of the oxidation.

The introduction of a catechol function at C_6 position of the imidazolopyrazinone moiety of compound **3a** leading to compound **3g** increased significantly the lag time ($p < 0.001$). Compound **3l**, characterised by a catechol and a *p*-hydroxyphenyl functions at C_6 and C_8 positions of

TABLE V Elemental analyses of tested compounds

Compound	Formula	Calc. (C, H, N, Cl)	Found (C, H, N, Cl)
1b	$C_{10}H_8N_2O$	69.75; 4.68; 16.23	69.63; 4.72; 16.06
1c	$C_{11}H_{10}N_2O$	70.95; 5.41; 15.04	70.96; 5.24; 15.08
1′f	$C_{11}H_{11}N_3O$	65.66; 5.51; 20.88	65.32; 5.34; 20.56
1f	$C_{10}H_9N_3O$	64.16; 4.85; 22.45	63.91; 4.77; 21.89
1′g	$C_{11}H_{11}N_3O$	65.66; 5.51; 20.88	65.46; 5.45; 20.66
1g	$C_{10}H_9N_3O$	64.16; 4.85; 22.45	64.70; 4.64; 21.91
1k	$C_{17}H_{15}N_3O$	73.57; 5.40; 11.13	73.90; 5.19; 11.45
1l	$C_{18}H_{17}N_3O.0.15H_2O$	73.46; 5.88; 14.28	73.37; 5.97; 13.86
2b	$C_{16}H_{12}N_2O_2$	72.72; 4.57; 10.60	71.95; 4.52; 10.54
2c	$C_{18}H_{16}N_2O_2$	73.95; 5.51; 9.58	73.61; 5.53; 9.51
2e	$C_{16}H_{13}N_3O$	72.99; 4.98; 15.96	72.45; 5.02; 15.64
2f	$C_{17}H_{15}N_3O$	73.63; 5.45; 15.15	73.03; 5.37; 14.98
2g	$C_{16}H_{13}N_3O$	72.99; 4.98; 15.96	72.33; 5.11; 15.55
2h	$C_{17}H_{15}N_3O$	73.63; 5.45; 15.15	73.25; 5.38; 15.01
2o	$C_{23}H_{19}N_3O_2.0.5H_2O$	72.93; 5.28; 11.09	72.57; 5.68; 10.28
2p	$C_{25}H_{23}N_3O_2$	75.50; 5.79; 10.57	75.46; 5.78; 10.41
3e	$C_{13}H_{11}N_3O_2.HCl.H_2O$	52.75; 4.73; 14.20; 12.00	52.62; 5.39; 14.82; 11.51
3f	$C_{13}H_{11}N_3O_2.HCl.0.5H_2O$	54.41; 4.53; 14.64; 12.38	54.26; 4.56; 14.66; 13.48
3h	$C_{19}H_{15}N_3O_2.HCl.1.2H_2O$	60.73; 4.90; 11.18; 9.45	60.72; 5.06; 10.67; 10.11
3i	$C_{19}H_{15}N_3O_2.HCl.1.2H_2O$	60.73; 4.90; 11.18; 9.45	60.55; 5.03; 10.76; 10.05
3k	$C_{21}H_{19}N_3O_3.HCl$	63.39; 5.07; 10.56; 8.91	62.40; 4.99; 10.24; 9.54

the imidazolopyrazine moiety, respectively, was as active as compound **3g**. No significant differences were observed between the catechol-substituted AMPs and their corresponding IMPZ at all tested concentrations (Table V). As for the 2,6-diamino-3,5-diaryl substituted AMPs (compounds **2j–l**), some of these compounds (e.g. **2j**) exhibited greater activity against lipid peroxidation than EGCG, vitamin E and trolox.

DISCUSSION

The ability of CLM to reduce the rate of the lipid peroxidation process requires the presence of both C_2-amino and C_5-phenol groups at the para position of the pyrazine core. While the removal of the phenol completely cancelled all chain-breaking properties, that of the amino group markedly reduced the antioxidant efficiency of the pyrazine. The inability of most aminopyrazines to completely block the onset of the peroxidation while they affect its rate of propagation is surprising. This suggests that while aminopyrazines react with AAPH-derived peroxyl radicals, some reaction product could have pro-oxidant effects on linoleate. It is possible that semiquinone radical likely to result from proton abstraction from the phenol substituent of the aminopyrazines could be responsible for this effect. However, the interpretation of antioxidant mechanisms from such system is very complex and depends on the nature of the lipid, the emulsifier and the free radical inducer.[19] It is also possible that the absence of lag phase reflects the occurrence of similar rates of the reactions of $ROO^{\bullet}$ radicals with the lipids and the aminopyrazines. By contrast, side-groups associated to the imidazolopyrazinone core have little influence on the ability of IMPZ to delay the onset of the oxidation. Thus the chain-breaking properties of IMPZ are most probably associated with the previously described reactivity of the imidazolone ring where two moles of peroxyl radicals react with one mole of IMPZ.[3,7,16] However the presence of phenol substituents at R1 and/or R2 positions of the IMPZ allowed some inhibition of the propagation rate after the induction period. Since the onset of the peroxidation appears to coincide with the consumption of the IMPZ, the reduced oxidation rate could possibly be ascribed to the production of some oxidation product with antioxidant properties. Since the amino-pyrazines reduce the propagation rate of the peroxidation, it is likely that they account for a part if not all of the slower peroxidation rate observed in phenol-substituted IMPZ. Surprisingly, *p*-amino-phenol which also possesses amino- and hydroxyl-groups located in para position has no effect on the rate but induces a lag time, suggesting that this could be either due to the pyrazine ring or the occurrence of two conjugated rings. It is particularly interesting that similar effects were observed on copper-induced LDL oxidation (not shown), that is, compounds **1a** and **2a** do not prolong the lag phase but slow the propagation of the oxidation while IMPZ induced a latency period. In order to determine the causes of this phenomenon EPR studies of radicals formed upon the oxidation of the compounds are currently carried out. It is likely that the stoichiometry of the reaction between mono-aminopyrazines is 2 such as with other phenols and therefore not different from that of the imidazolopyrazinone.

Best activities were obtained through the replacement of the phenol group with a catechol substituent in both families (compounds **1h**, **2m** and **2n**; **3g** and **3l**). Interestingly catechol-substituted AMP derivatives induced a lag time similarly to IMPZ derivatives. This suggests that catechol substituents suppress the ability of the semiquinonic radical to further propagate the oxidation process. This is probably achieved by intramolecular hydrogen bonding of the aryloxyl radical semiquinone such as in other catechols.[20]

The efficiency of catechol-substitued AMP's (compounds **1h** and **2n**) was similar to that of corresponding IMPZs (compounds **3g** and **3l**). The introduction of a second amine in R4 position (compound **2j**) and a second phenol in R2 of the aminopyrazines markedly increased the chain breaking properties. Interestingly, this compound induced both a lag phase and a reduced rate of oxidation. Substituting the two phenols by methoxy groups cancelled the rate limiting effect but not the induction period. According to the above hypothesis that the aryloxyl radical semiquinone formed upon hydrogen abstraction of phenol-substituted amino-pyrazines is responsible for reduced rate of peroxidation and the absence of a lag phase, this could indicate that the occurrence of two amino groups better stabilize the semiquinone radicals and prevent their reaction with linoleate. There are no previous report of antioxidant properties in 2,6-diaminopyrazines in the literature. On the other hand, 2,6-diaminopyridine has been proposed as heat-resistant antioxidant for use in hydraulic fluids.[21] While it is very likely that 2,6-diamino-pyridine and 2,6-diaminopyrazines share the same antioxidant mechanism, the stoichiometry of their reaction remains unknown.

In conclusion, both catechol-substituted amino-pyrazines and 2,6-diaminopyrazines are highly efficient antioxidants, with lag phase longer than those induced by reference antioxidants such as vitamin E, trolox and epigallocatechin gallate. Also, these data confirm the high antioxidative properties of imidazolopyrazinone compounds. They also show that CLM can yield derivatives more efficient than reference antioxidants such as EGCG.

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