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Preliminary communication

Synthesis and antioxidant activity of new tetraarylpyrroles

Jacques Lehuédé[a], Bernard Fauconneau[b], Laurence Barrier[b], Marina Ourakow[a], Alain Piriou[b], Jean-Michel Vierfond[a]*

[a]*Laboratoire de Chimie Organique, Faculté de Pharmacie, BP 199, 34, rue du Jardin des Plantes, F-86005 Poitiers, France*
[b]*Centre d'Études et de Recherche sur les Xénobiotiques, EA 1223, Faculté de Pharmacie, BP 199, 34, rue du Jardin des Plantes, F-86005 Poitiers, France*

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Abstract – The synthesis and in vitro antioxidant activity of 17 new tetraarylpyrroles are investigated by 2 tests highly documented in the literature: capability to prevent Fe^{2+}-induced lipid peroxidation on microsomes, which is a membrane preparation rich in polyunsaturated fatty acids, and direct scavenging effect on a stable free radical, 1,l-diphenyl-2-picryl-hydrazyl (DPPH). For the Fe^{2+}-induced microsomal lipid peroxidation system, the results show that molecules which possess 2-pyrazinyl or 2-pyridyl in the 3- and 4-positions on the pyrrole ring are the most efficient. Introduction of methoxy groups on the phenyl ring in the 2- and 5-positions increases the effects but the higher activity is obtained with 2-furyl or 2-thienyl. The only compounds which possess a direct scavenger effect on trapping the stable free radical DPPH are those which have 2-pyridyl in the 3- and 4-positions and 2-furyl or 2-thienyl in the 2- and 5-positions. © 1999 Éditions scientifiques et médicales Elsevier SAS

tetraarylpyrrole / antioxidant activity / radical scavenging effect / microsomes / DPPH

1. Introduction

Formation of reactive oxygen species and the ensuing oxidation of biological molecules is a well recognized mechanism of tissue damage in many pathological situations, such as, inflammation, stroke, acute myocardial infarction and the subsequent reperfusion phase. Numerous natural or synthetic antioxidant compounds have been tested with success in various disease models as well as in clinics [1].

In the literature, it is well known that pyrrolic compounds such as polypyrroles, poly- or hetero-arylpyrroles present an electronic delocalization, conferring to these molecules electric conductor and oxidizable properties [2, 3]. Furthermore, pyrrolic structures such as benzoylpyrrole-3-acetic acids were tested in vitro by examining their effects on lipid peroxidation using rat hepatic microsomes and as hydroxyl radical scavengers [4].

*Correspondence and reprints

Recently we reported results on six pyrrolic compounds synthesized in our laboratory showing interesting antioxidant activities [5]. Consequently, it seems relevant to test large series of molecules bearing different substituents on the pyrrole ring: phenyl rings (with or without methoxy groups) and aromatic heterocycle rings in order to have an electronic delocalization and consequently, potent antioxidant activities.

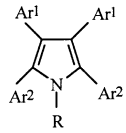
2. Chemistry

The synthesis of compounds **2a–q** (*table I*) was realized in two steps (*figure 1*): metalation of methylazine from lithium diisopropylamine and condensation with an aromatic nitrile to obtain an imine-enamine **1** moisture sensitive (first step), and oxidation of imine-enamine **l** by $Pb(OAc)_4$ to give tetraarylpyrroles **2** (second step) [6]. The structure is given in *table I*.

2.1. Antioxidant activity

The antioxidant potency of compounds **2a–q** was assessed by two tests commonly used in the literature

Table I. Structure and antioxidant effects of tetraarylpyrroles. IC_{50} values are expressed as means ± SEM.



	R	Ar^1	Ar^2	Yields (%)	IC_{50} microsomes (μM)	IC_{50} DPPH (μM)
2a	H	2-pyrazinyl	phenyl	90[a]	320 ± 16	> 1 000
2b	H	2-pyrazinyl	2-pyridyl	77[a]	> 1 000	> 1 000
2c	H	2-pyrazinyl	4-pyridyl	52[a]	> 1 000	> 1 000
2d	H	2-pyrazinyl	2-furyl	98[a]	19.0 ± 9.6	> 1 000
2e	H	2-pyrazinyl	2-thienyl	90[a]	8.0 ± 1.1	> 1 000
2f	H	2-quinoxalinyl	phenyl	59[a]	> 1 000	> 1 000
2g	H	2-pyridyl	phenyl	50[b]	67.2 ± 6.7	> 1 000
2h	H	4-pyridyl	phenyl	37[b]	> 1 000	> 1 000
2i	H	2-pyridyl	2-methoxyphenyl	53[c]	28 ± 3	> 1 000
2j	H	2-pyridyl	3-methoxyphenyl	36[c]	63 ± 4	> 1 000
2k	H	2-pyridyl	4-methoxyphenyl	67[c]	12.3 ± 4.0	> 1 000
2l	H	2-pyridyl	3,4,5-methoxyphenyl	25[c]	26.2 ± 8.5	> 1 000
2m	H	4-pyridyl	4-methoxyphenyl	29[c]	100 ± 12	> 1 000
2n	H	2-pyridyl	2-furyl	17.5[b]	11.0 ± 1.9	200 ± 50
2o	CH_3	2-pyridyl	2-furyl	88[d]	61.1 ± 5.0	> 1 000
2p	H	2-pyridyl	2-thienyl	53[b]	2.7 ± 1.1	410 ± 127
2q	H	2-pyridyl	3-thienyl	32[b]	12.5 ± 2.2	> 1 000
Trolox					5.0 ± 0.3	10.1 ± 0.5

[a]: based on isolated imine-enamine. [b]: based on aromatic nitrile. [c]: with TMEDA and heating, based on aromatic nitrile. [d]: methylation from **2n**.

bearing on this topic: capability to prevent Fe^{2+}-induced lipid peroxidation on microsomes, which is a membrane preparation rich in polyunsaturated fatty acids, and direct scavenging effect on a stable free radical, 1,l-diphenyl-2-picryl-hydrazyl (DPPH).

The results were compared to those observed with Trolox (water soluble vitamin E analogue), a reference antioxidant.

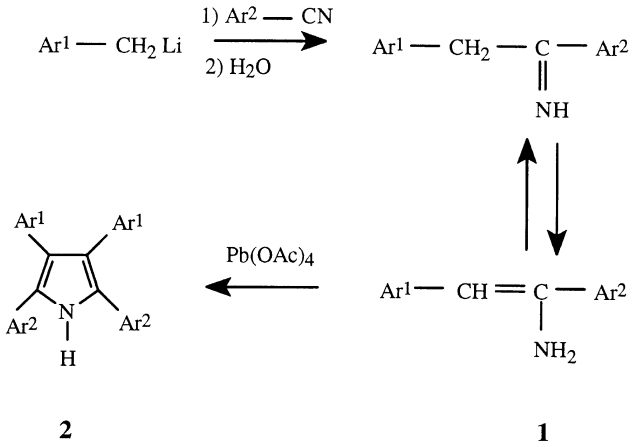


Figure 1. Synthesis of tetraarylpyrroles.

3. Results and discussion

3.1. Effect on lipid peroxidation

Three distinctive steps can be distinguished in lipid peroxidation: initiation, propagation and termination reactions. Lipid peroxidation may be induced, for example, by radical species which are sufficiently reactive to abstract a hydrogen atom from the unsaturated fatty acids. This is the starting point for the lipid radical chain propagation reaction. Thus, many molecules of lipids may be oxidized to lipid hydroperoxides for every initiation event. The propagation cycle is broken by termination reactions which result in the destruction of free radicals. Termination reactions occur when two radical species combine to form non-radical final products [7].

A chain breaking antioxidant has the ability to donate hydrogen radicals and contributes to stop the chain reaction. It reduces the primary radical by a one electron reduction to a non-radical chemical species and, as a consequence, is transformed into an oxidized antioxidant radical. However, this antioxidant must be more than just a hydrogen atom donor; it needs also to form a radical of such low reactivity that no further reactions with lipids can occur.

Peroxyl radicals are the major chain-propagating species in the process of lipid peroxidation in membranes [8]. Thus, a standard test for antioxidants is the action of a substance in inhibiting peroxidation of membranes such as microsomes. The thiobarbituric acid (TBA) test is widely used in microsomal systems but it is essential to ensure that the apparent antioxidant effect of an added compound is not due to the interference with the TBA test itself [9]. Consequently, relevant controls are necessary to check the absence of such an interference. Peroxidation can be accelerated by adding iron salts to membranes, e.g. Fe^{2+}. In metal-ion dependent systems, an added antioxidant might act not only by scavenging peroxyl radicals but also by binding iron ions and stopping them from accelerating peroxidation. However, these two possibilities can be distinguished easily. The efficient compounds tested in this study acted as chain breaking antioxidants because at low concentrations, a lag period into the peroxidation process was observed (data not shown), corresponding to the time taken for the antioxidant to be consumed, whereas metal-binding antioxidants give a constant inhibition throughout the reaction [9].

The effects of the compounds on lipid peroxidation induced by $FeCl_2$ are shown in *table I*. When Ar^2 is constant, i.e. phenyl ring, and if Ar^1 is 2-pyridyl (**2g**), the activity of the molecule is higher than that observed with Ar^1 = 2-pyrazinyl (**2a**). The activity disappears if Ar^1 is 4-pyridyl (**2h**) or 2-quinoxalinyl (**2f**). If the phenyl ring in the Ar^2 position is substituted, i.e. 4-methoxyphenyl, the higher activity of 2-pyridyl compared with that of 4-pyridyl in Ar^1 is confirmed (**2k** versus **2m**). When they are in the Ar^1 position, the tested substituents can be classified as follows: 2-pyridyl > 2-pyrazinyl > 4-pyridyl > 2-quinoxalinyl. Consequently, molecules with Ar^1 = 2-pyridyl or 2-pyrazinyl show the greatest interest and the synthesis of molecules with different groups in Ar^2 positions is performed to improve the antioxidant effect.

When methoxy groups are added in the 2, 3, or 4 position on the phenyl ring in Ar^2, the antioxidant activity is increased (**2i**, **2j**, **2k** versus **2g**), with a major activity for 4-methoxyphenyl (**2k**). This activity may be correlated to the introduction of electron donor substituents which stabilize the generated radical during oxidation. The electronic mesomeric donor effect is of the same type when methoxy groups are added in the 2 or 4 position on the Ar^2 phenyl ring (i.e. **2i** or **2k**), but **2k** has a better activity, probably due to the steric bulk observed with 2-methoxyphenyl (**2i**). The substituent 3-methoxyphenyl has a lower electronic effect, and consequently is less active than **2i** and **2k**. But methoxy tri-substitution in the 3, 4, 5 positions on the phenyl ring (**2l**) decreases 2 times the activity of **2k**.

The antioxidant tests performed with compounds bearing heterocyclic substituents in the Ar^2 position make it possible to classify them into two groups: those with 2- and 4-pyridyl which are inactive (**2b** and **2c**) and those with pentagonal heterocycles (**2d**, **2e**, **2n**, **2p**, **2q**) which seem to be of major importance because the antioxidant activity is at least as great as that of the more active phenylmethoxylated compounds. The pentagonal substituents have a donor mesomeric effect on a conjugated system, whereas pyridyl has an attractor effect. Substituent 3-thienyl (**2q**) is less active than 2-thienyl (**2p**).

On microsomes oxidized by Fe^{2+}, **2p** (Ar^1 = 2-pyridyl and Ar^2 = 2-thienyl; IC_{50} = 2.7 ± 1.1 μM) is the most efficient antioxidant. Its activity is higher than that obtained with Trolox (5.0 ± 0.3 μM), a reference antioxidant.

These results show the major importance of the pyrrol ring in antioxidant effects since methylation of **2n** (i.e. **2o**) decreases about 6 times the activity of this compound. This may be explained by the difficulty in forming a free radical with a pentasubstituted pyrrole.

3.2. Effect on DPPH

The DPPH test provided information about the reactivity of the tested compounds with a stable free radical. Because of its odd electron, the DPPH radical showed a strong absorption band at 515 nm in visible spectroscopy (a deep purple colour). As this electron is paired off in the presence of a free radical scavenger, absorption vanishes and the resulting decoloration is stoichiometric with respect to the number of electrons taken up. This bleaching of DPPH absorption, which occurs when the odd electron of the radical is paired, is thus representative of the capacity of the compounds to scavenge free radicals independently of any enzymatic activity.

Only **2p** and **2n** have a radical scavenging activity, with a major activity for **2n**, whereas that of Trolox is considerably higher. It means that these compounds possess a direct trapping effect (*table I*).

It is noteworthy that these molecules (i.e. **2p** and **2n)** also have a good activity with a microsome system.

Nevertheless, **2e** has no activity on DPPH, while it has good activity on Fe^{2+}-induced microsomal lipid peroxidation. This result shows that this molecule doesn't possess a direct scavenger effect, at least on this radical. This effect is improved when a 2-pyridyl is substituted in the Ar^1 position (**2e** versus **2p**).

In conclusion, our study provides evidence that several tetraarylpyrroles exhibit interesting antioxidant properties mainly expressed by their capacity to inhibit Fe^{2+}-induced microsomal lipid peroxidation. These effects may be useful in the treatment of pathologies in which free radical oxidation plays a fundamental role.

The early trials on animals are encouraging, since toxicity tests have been performed on female Swiss mice (18–20 g, Iffa-Credo, l'Arbresle, France). At the present time, the only compound tested is **2a** and is responsible for no mortality when orally administered at the dose of 1g/kg (batch of 10 animals).

4. Experimental protocols

Melting points were measured by using a Köfler apparatus and are uncorrected. The ^{1}H-NMR spectra were recorded on Varian EM 360 and Bruker 200 A.C. spectrometers. IR spectra were realized on a Unicam SP 1100 and on an ATI Mattson Genesis spectrometer. Elemental analyses were performed on a Perkin Elmer 240 apparatus.

Acetonitrile was dried over Na_2SO_4. Tetrahydrofuran (THF) was dried and extemporaneously distillated over sodium. Diisopropylamine and tetramethylethylenediamine (TMEDA) were dried over BaO and distillated. Butyl lithium used was a Merck 1.6 M solution in hexane.

The synthesis of compounds **2a–h** was previously described [6]. The **2n–q** pyrroles were prepared according to the precedent method without isolation of the imine-enamine intermediate.

*4.1. General procedure for the preparation of methoxylated phenylpyrroles (***2i–m***)*

Imines were prepared according to the general procedure [6]. 13.8 mL (22 mmol) of a solution of n-butyllithium (1.6 M) in hexane was added, via syringe, to a stirred solution of diisopropyl-amine (2.2 g; 22 mmol) solved in 30 mL of dry THF plus 10 mL of dry TMEDA, under 0 °C nitrogen atmosphere. After 20 min at 0 °C, the solution was cooled to –40 °C and a solution of 2-methylpyrazine (1.88 g; 20 mmol) in 5 mL of THF was slowly added. Then, the solution was stirred for 45 min at –40 °C and a solution of methoxylated aromatic nitrile (10 mmol) in 5 mL of mixed solvent (THF 3/4 plus TMEDA 1/4; v/v) was added at –40 °C. The mixture was heated for 2 h to refluxing THF afterwards. After cooling to 20 °C, 1 mL of water was added, with strong and quick stirring, the mixture was stored on anhydrous sodium sulphate. After filtration and evaporation of solvents, the residue (non-isolated imine-enamine) was dissolved in acetonitrile (100 mL) plus chloroform (50 mL) and cooled to –40 °C. The lead tetraacetate (2.22 g; 5 mmol) was introduced at –40 °C with stirring. After 15 min, the mixture was heated to 20 °C and stirred for 1 h. Then, 12 mL of aqueous saturated sodium carbonate was added. The precipitate was washed out with acetonitrile and chloroform. The organic phase was dried over anhydrous sodium sulphate, evaporated and purified by chromatography on a silica gel column, elution with ethyl acetate.

4.1.1. 1H-2.5-di-(2-methoxyphenyl)-3.4-di-(2-pyridyl) pyrrole **2i**

Yellow powder (2.29 g, 53%); m.p. 179 °C; IR (KBr) (cm^{-1}): 3 480, 1 580, 783, 751; ^{1}H-NMR ($CDCl_3$) δ 10.2 (1H, s, NH), 8.4 (2H, d, *J* = 4–5 Hz), 7.5–6.6 (14H, m), 3.7 (6H, s); Anal. $C_{28}H_{23}N_3O_2$ (C, H, N).

4.1.2. 1H-2.5-di-(3-methoxyphenyl)-3.4-di-(2-pyridyl) pyrrole **2j**

Pale yellow powder (1.55 g, 36%); m.p. 165 °C; IR (KBr) (cm^{-1}): 3 389, 1 608, 1 558, 871, 852, 784, 748; ^{1}H-NMR ($CDCl_3$) δ 8.8 (1H, s, NH), 8.45 (2H, m), 7.5–6.6 (14H, m), 3.6 (6H, s); Anal. $C_{28}H_{23}N_3O_2$ (C, H, N).

4.1.3. 1H-2.5-di-(4-methoxyphenyl)-3,4-di-(2-pyridyl) pyrrole **2k**

Pale yellow powder (2.90 g, 67%); m.p. 128 °C; IR (KBr) (cm^{-1}): 3 210, 1 590, 834, 792, 744; ^{1}H-NMR ($CDCl_3$) δ 8.7 (1H, s, NH), 8.4 (2H, d, *J* = 4–5 Hz), 7.5–6.6 (14H, m), 3.7 (6H, s); Anal. $C_{28}H_{23}N_3O_2$ (C, H, N).

4.1.4. 1H-2.5-di-(3,4,5-trimethoxyphenyl)-3,4-di-(2-pyridyl) pyrrole **2l**

Brown powder (1.39 g, 25%); m.p. > 260 °C; IR (KBr) (cm^{-1}): 3 357, 1 584, 842, 793, 747, 699; ^{1}H-NMR ($CDCl_3$) δ 9.0 (1H, s, NH), 8.5 (2H, d, *J* = 4–5 Hz), 7.6–6.9 (6H, m), 6.6 (4H, s), 3.8 (6H, s), 3.65 (12H, s); Anal. $C_{32}H_{31}N_3O_6$ (C, H, N).

4.1.5. 1H-2.5-di-(4-methoxyphenyl)-3,4-di-(4-pyridyl) pyrrole **2m**

White powder (1.25 g, 29%); m.p. > 260 °C; IR (KBr) (cm^{-1}): 3 350, 1 550, 900, 645; ^{1}H-NMR ($CDCl_3$) δ 8.75 (1H, s, NH), 8.2 (4H, d, *J* = 4–5 Hz), 7.15–6.6 (12H, m), 3.7 (6H, s); Anal. $C_{28}H_{23}N_3O_2$ (C, H, N).

4.1.6. 1H-2.5-di-(2-furyl) 3,4-di-(2-pyridyl)pyrrole **2n**

Pale yellow powder (0.62 g, 17.5%); m.p. 172 °C; IR (KBr) (cm^{-1}): 3 430, 1 589, 1 562, 803, 790, 733; ^{1}H-NMR ($CDCl_3$) δ 9.4 (1H, s, NH), 8.6 (2H, m), 7.5–6.9 (8H, m), 6.3 (4H, m); Anal. $C_{22}H_{15}N_3O_2$ (C, H, N).

4.1.7. 1H-1-methyl-2.5-di-(2-furyl)-3,4-di-(2-pyridyl) pyrrole **2o**

Brown powder; m.p. 180 °C; IR (KBr) (cm^{-1}): 3 134, 1 587, 1 561, 909, 750; ^{1}H-NMR ($CDCl_3$) δ 8.4 (2H, m), 7.5–6.8 (8H, m), 6.3 (4H, m), 3.6 (3H, s); Anal. $C_{23}H_{17}N_3O_2$ (C, H, N).

4.1.8. 1H-2.5-di-(2-thienyl)-3.4-di-(2-pyridyl)pyrrole **2p**

Pale yellow powder (2.04 g, 53%); m.p. 230 °C; IR (KBr) (cm^{-1}): 3 250, 1 590, 840, 750, 690; ^{1}H-NMR ($CDCl_3$) δ 8.7 (1H, s, NH), 8.5 (2H, m), 7.5–6.8 (12H, m); Anal $C_{22}H_{15}N_3S_2$ (C, H, N).

4.1.9. 1H-2.5-di-(3-thienyl)-3.4-di-(2-pyridyl)pyrrole **2q**

Pale yellow powder (1.23 g, 32%); m.p. 243 °C; IR (KBr) (cm^{-1}): 3 420, 1 590, 1 560, 865, 782, 749, 693, 616; ^{1}H-NMR ($CDCl_3$) δ 9.6 (1H, s, NH), 8.4 (2H, m), 7.5–6.8 (12H, m) Anal. $C_{22}H_{15}N_3S_2$ (C, H, N).

4.2. Procedures concerning antioxidant activities

4.2.1. Chemicals

All chemicals were purchased from the Sigma Chemical Co. (St. Louis, MO, USA) except Trolox which was obtained from Aldrich (St Quentin Fallavier, France).

The powders corresponding to the molecules **2a–q** were kept under nitrogen. For the biological tests, the compounds were dissolved extemporaneously in ethanol and the different dilutions were performed in ethanol.

4.2.2. Inhibitory effect on lipid peroxidation

Male Sprague-Dawley rats (200–250 g, Iffa-Credo, l'Arbresle, France) had been deprived of food overnight (16 h). The rats were anaesthetized by inhalation of ethyl ether. Livers were perfused, rapidly isolated and minced thoroughly with scissors. The minced tissue was washed with ice-cold 0.15 M KCl and homogenized with 5 volumes of ice-cold 0.15 M KCl, using a Teflon-glass potter homogenizer (3 000 rpm for 2 min). Microsomal fractions were isolated in Tris-HCl 0.05 M/KCl 0.15 M, pH 7.4, by removal of the nuclear fraction at 800 *g* for 15 min, removal of the mitochondrial fraction at 15 000 *g* for 15 min and sedimentation at 105 000 *g* for 30 min. Pellets were washed twice in Tris-HCl 0.05 M/KCl 0.15 M, pH 7.4 buffer by centrifugation, with subsequent sedimentation at 125 000 *g* for 15 min. Microsome pellets were resuspended in the same buffered solution at 5 mg protein/mL and stored at –80 °C for a maximum of 1 month. The protein content was determined by the method of Bradford [10], using bovine albumin as a standard.

For the test, microsomal fractions were thawed just before use and were diluted with 0.05 M Tris-HCl, pH 7.4, containing 0.15 M KCl. The final protein concentration in the incubation mixture amounted to 0.75 mg/mL. Microsomes were pre-incubated with different concentrations of compounds in a shaking water-bath at 37 °C for 10 min. Then, lipid peroxidation was initiated with 10 μM $FeCl_2$ and the samples were incubated at 37 °C for 30 min. After action of thiobarbituric acid, the absorbance was measured at 532 nm for thiobarbituric acid reactive substance (TBARS) determination [11], versus control containing the same quantity of ethanol but without the compound studied. The measurements were performed in triplicate. The inhibition of lipid peroxidation was expressed as a percentage and the inhibition concentration 50% (IC_{50}) was obtained from the inhibition curve by graphical determination.

4.2.3. Radical scavenging effect

Free radical scavenging capacity of the compounds was determined using DPPH [12]. An ethanol DPPH solution (0.1 mM) was mixed with different concentrations of compound and the absorbance change at 515 nm was measured 10 min later with a spectrophotometer (Uvikon 940, Kontron) versus control containing the same quantity of ethanol, but without compound studied. The measurements were performed in triplicate. The inhibition of coloration was expressed as a percentage and the IC_{50} was obtained from the inhibition curve, by graphical determination.

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