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SYNTHESIS OF NOVEL *ORTHO*ALKYLAMINOPHENOL DERIVATIVES AS POTENT NEUROPROTECTIVE AGENTS *IN VITRO*.

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Abstract : A series of *ortho*alkylaminophenol derivatives was synthesized and tested *in vitro* with respect to their neuroprotective effect. Some of these compounds exhibited a potent antioxidant activity close to that of standard α-tocopherol.

Keywords : Antioxidants, Neuroprotective agents, Radical scavengers, Substituent effects.

Reactive oxygen species (e.g. superoxide, hydrogen peroxide and hydroxyl radicals) are produced continuously in living cells, and various defence systems are available within cells and tissues to prevent or minimize free-radical-induced damage. However, in acute situations, reactive oxygen species-mediated damage can overwhelm normal cellular defence systems. Disturbance in the prooxidant-antioxidant balance, in favour of the former, lead to a widely accepted phenomenon called "oxidative stress".[1] In pathological aging, oxidative stress may play a role in the progression of many neurodegenerative pathologies of the central nervous system, including Parkinson's and Alzheimer's Disease. This has prompted the search for efficient antioxidants which can provide defence against a stress imposed by severe pathological conditions.

In this context, exifone (*Adlone*^{R}) was launched in France for the treatment of cognitive disorders in the elderly,[2,3] but was withdrawn rapidly from the market because of its hepatotoxic effects. Mainly based on previous results concerning the possible role of highly electrophilic oxidation products in the toxicological effects of drugs, [4-6] we hypothesized that a potential toxic metabolite of exifone could be the transient reactive *ortho*quinone species, that would bind irreversibly to the NH_2 and SH residues of cellular proteins to form conjugates.[7-9] Our objective was then to scavenge the transient reactive *ortho*quinone by the formation of an adduct blocking the electrophilic sites generally attacked by membrane nucleophiles, and consequently to prevent toxicity.

A few years ago, we described the first electrochemical and chemical syntheses of novel 1,4-benzoxazine derivatives structurally related to exifone [eqn. (2), scheme 1], that have been identified as efficient antioxidants and significantly less hepatotoxic than exifone.[10] The key step consisted of the reaction of the transient electrogenerated 3,4-quinone [eqn. (1)] with aminoalcohols [CH_2OH - $C(R^1, R^2)$ - NH_2] at the 2-position.[11] By varying the nature of the aminoalcohol, we established that neither R^1 nor R^2 could be an hydrogen atom. Nevertheless, in the presence of aminoalcohols derived from natural aminoacids (CH_2OH -

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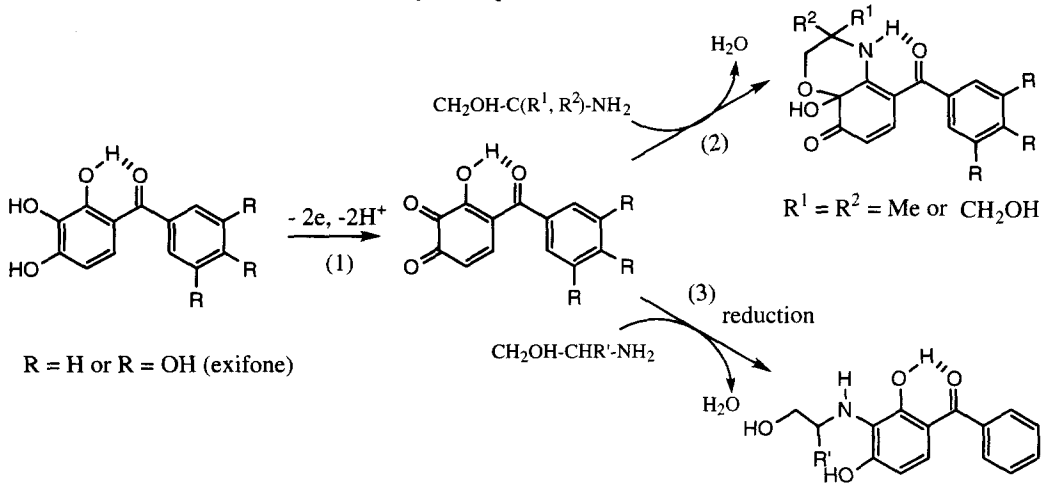
CHR'- NH_2), a competing reaction arose implying a nucleophilic attack of the transient electrogenerated 3,4-quinone species at the 3-position [eqn. (3)] rather than at the 2-position [eqn. (2)]. This reaction led, after electrochemical reduction, to novel 3,4-alkylaminophenol derivatives.[12,13]



Scheme 1

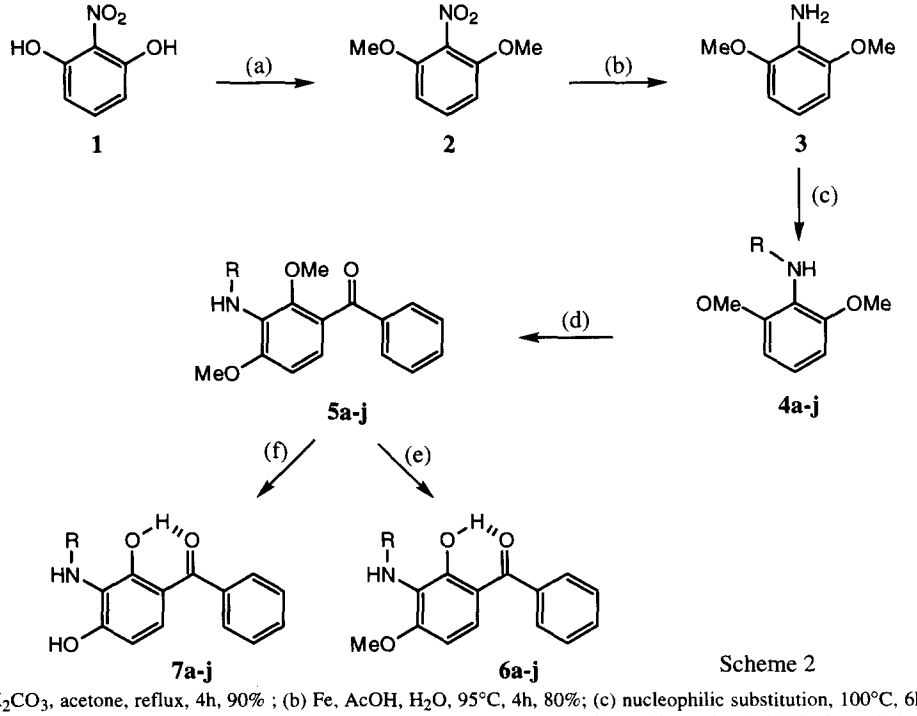
In the course of our investigations aimed at the preparation of molecules of pharmacological interest, we focused our attention on this competing reaction that could be regarded as another way to scavenge the transient unstable 3,4-quinone, and consequently to reduce toxicity. Preliminary *in vitro* evaluation performed on several electrochemically prepared 3,4-alkylaminophenols[12] revealed that some of these compounds exhibited a significant antioxidant activity. Consequently, we thought that subsequent investigations could be worthwhile to assess the utility of these compounds as antioxidant agents.

In this paper, we describe the chemistry as well as the results of *in vitro* assays concerning a series of variously substituted *ortho*alkylaminophenols. Redox potentials have been deduced from electrochemical measurements to evaluate if the requirements for a molecule to be an efficient antioxidant are predominantly influenced by its redox properties.

Chemistry

Preliminary *in vitro* results suggested the possibility that other potentially useful structural modifications might be of interest. So, we need to easily synthesize small to large quantities of various *ortho*alkylaminophenol derivatives. Toward this goal, we attempted to prepare these compounds from commercially available 2-nitroresorcinol **1** *via* a five step sequence which involved protection and deprotection steps as reported in scheme 2. Alkylation of 2-nitroresorcinol **1** with MeI and K_2CO_3, followed by reduction of the nitro group with Fe powder under acetic acidic conditions,[14] afforded **3** in roughly 75% overall yield. The latter could be N-alkylated following two alternative methods depending on the nature of the alkyl chain that had to be incorporated. The first method was nucleophilic substitution of **3** with appropriate primary alkyl halides in the absence of solvent, leading to compounds **4b**, **4c** and **4h-j** in moderate yields (50%). The second method utilized reductive alkylation with sodium triacetoxyborohydride in 1,2-dichloroethane,[15] starting from appropriate ketone or aldehyde and substituted aniline **3**. Compounds **4a-j** were then obtained in good yields

ranging from 60 to 80%. Polyphosphoric acid was selected as a catalyst in the subsequent Friedel-Crafts acylation reaction[16] with benzoic acid affording compounds **5a-j**. In addition to the simplicity of the method, the absence of ether cleavage products made the reaction the one of choice for this type of compound. The usual Friedel-Crafts procedure using an acid chloride in conjunction with aluminum chloride as a catalyst invariably resulted in partial or total ether cleavage when the acyl function entered *ortho* to the methoxy group. Finally, synthesis of *ortho*alkylaminophenol derivatives **6a-j** and **7a-j** could be achieved through demethylation of aryl methyl ethers. Regiospecific monodemethylation at the 2-position of the benzophenone skeleton was successfully performed using 5 equiv of $AlCl_3$ with boiling dichloromethane, to afford compounds **6a-j** in good yields ranging from 70 to 90%. Treatment of compounds **5a-j** with 5 equiv of $AlCl_3$ in toluene heated at reflux, or alternatively with 5 equiv of BBr_3 in dichloromethane at room temperature, caused complete deprotection to compounds **7a-j** (45-60% yields).



Scheme 2

(a) MeI, K_2CO_3, acetone, reflux, 4h, 90% ; (b) Fe, AcOH, H_2O, 95°C, 4h, 80%; (c) nucleophilic substitution, 100°C, 6h, 50% or reductive alkylation, $NaBH(OAc)_3$, AcOH, 1,2-dichloroethane, room temperature, 6h, 60-80% ; (d) Friedel-Crafts acylation, benzoic acid, polyphosphoric acid, 80°C, 8h, 45-55% along with a noticeable amount of starting material **4a-j** (~ 45%) ; (e) $AlCl_3$, CH_2Cl_2, reflux, 1h, 70-90% ; (f) $AlCl_3$, toluene, reflux, 2h, 45-60% or BBr_3, room temperature, 3h, 60%.

In vitro biological results

The intrinsic neurotoxicity as well as the neuroprotective activity of *ortho*alkylaminophenol derivatives were assessed *in vitro* on murine HT-22 hippocampal cell cultures and compared with those of parent exifone as well as standard α-tocopherol.[17] The results are presented in Table.

In order to determine potential pro-oxidant effects and to select a concentration range lacking intrinsic toxicity suitable for studying the neuroprotective activity of the compound, the intrinsic neurotoxic effects of each compound was evaluated following two different methods. Neurotoxicity was monitored either by quantification

of cellular lysis (death) after measurement of the lactate dehydrogenase (LDH) activity released from damaged cells into the culture supernatant[20] or by reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT reduction assay),[21] that allows an evaluation of the "redox state" of the cells and emphazises oxidative stress. The maximum tolerated concentration (MTC), and the concentration producing 50% toxicity (TC_{50}), were estimated for each tested compounds using both MTT and LDH determination.[22]

Neuroprotective properties of *ortho*alkylaminophenol derivatives were estimated through their protective effects against L-homocysteic acid (L-HCA) cytotoxicity.[23] The latter has been reported to provoke, on immature primary neurons or on certain neuronal cell lines, oxidative-stress-mediated neuronal degeneration that could be attenuated with antioxidants.[24] The concentration leading to 50% protection (PC_{50}) was estimated for both MTT and LDH determinations.

As reported in Table, all tested compounds of this series showed significant *in vitro* neuroprotective activity, with PC_{50} (MTT) values between 2.6 and 15.6 μM and PC_{50} (LDH) values between 1.1 and 15.4 μM. Interestingly, most of the compounds were found to be more active than parent exifone ($PC_{50} > 10$ μM). Even, some of them exhibited neuroprotective activity close to that of standard α-tocopherol (see compounds **6g** and **7g** for example).

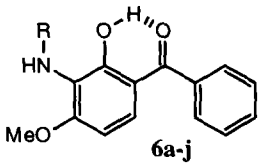
Using the MTT reduction assay, substituted 4-methoxy derivatives **6c**, **6f**, **6g** and **6j** seemed to be slightly less active than their substituted 4-hydroxy counterparts **7c**, **7f**, **7g** and **7j** with PC_{50} values of 15.6, 6.7, 3.1 and 5.9 μM compared to 8.5, 5.5, 2.6 and 4.1 μM respectively. This was not always the case using LDH determination assay, since compounds **6g** (PC_{50} = 1.1 μM) was found to be more potent than its counterpart **7g** (PC_{50} = 2.7 μM).

Within the most active sub-series of 4-hydroxy derivatives, structural modifications of the 3-alkylamino chain did not markedly affect neuroprotective activity, so that structure-activity relationships were not obvious. Replacement of the 3-alkylamino chain by mono or dihydroxyalkylamino chain induced a slight decrease in activity (less than 2-fold) : compare **7f** [PC_{50} (MTT) = 5.5 μM] with **7e** [PC_{50} (MTT) = 10.0 μM] or with **7d** [PC_{50} (MTT) = 8.9 μM]. Substituted 3-benzylamino derivative **7j** with PC_{50} (MTT) value of 4.1 μM was as active as 3-alkylamino derivatives **7f-i** with PC_{50} (MTT) values between 2.6 and 5.5 μM.

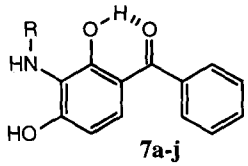
Within the sub-series of 3-alkylamino derivatives, substituted 3-n-propylamino derivatives **7h** [PC_{50} (MTT) = 5.1 μM] and 3-isopropylamino derivative **7f** [PC_{50} (MTT) = 5.5 μM] were equipotent. Replacement of the C-3 alkylamino substituent by more lipophilic C-5 alkyl groups [**7g**, PC_{50} (MTT) = 2.6 μM and **7i**, PC_{50} (MTT) = 4.0 μM] slightly enhanced activity. Accordingly, it should be noted that complete shortening of the 3-alkylamino chain to an amino group generally reduced activity. For example, compare **7g** with **7a** [PC_{50} (MTT) = 11.9 μM].

Our initial objective was to design a series of *ortho*alkylaminophenol derivatives that effectively protected neuronal cell lines *in vitro* from the toxicity induced by L-HCA, without causing any intrinsic toxic effects to the neurons. As expected, the MTC determined from the MTT reduction assay appeared to be the most sensitive parameter for all evaluated compounds. With regard to this parameter, with exifone we were unable to determine in our tests a concentration range for which antioxidant effects could be achieved without the manifestation of intrinsic toxicity (compare PC_{50} with MTC). In contrast, four compounds of the 3,4-alkylaminophenols series **7b**, **7d**, **7f** and **7h** exhibited decreased toxicity with MTC values, 2.5-5 fold higher than that of parent exifone. The results of both toxicity and activity were then combined to determine a safety index defined as MTC / PC_{50}. The latter could be used to estimate the therapeutic potential of the four selected compounds.

Table. *In vitro* biological activity of compounds **6a-j** and **7a-j**.



6a-j



7a-j

Compd	R	Toxicity				Protection *vs* 2mM L-HCA		Safety index
		MTC (µM)		TC_{50} (µM)		PC_{50} (µM)		MTC / PC_{50}
		MTT	LDH	MTT	LDH	MTT	LDH	MTT
α-tocopherol		>200	>200	>200	>200	0.8	1.7	>250
exifone		10	50	34	91	>10.0	>10.0	NR
6c	CH_2-CH_2OMe	50	50	146	186	15.6	15.4	3.2
6f	Pr^i	10	10	21	19	6.7	7.9	1.5
6g	$CH(Et)_2$	10	10	16	20	3.1	1.1	3.2
6j	Bzl	10	>250	78	>250	5.9	5.0	1.7
7a	H	25	25	60	75	11.9	15.3	2.1
7b	CH_2-CH_2OH	50	100	95	>250	8.5	8.0	5.9
7c	CH_2-CH_2OMe	10	>250	17	>250	8.5	6.6	1.2
7d	$CH(CH_2OH)_2$	25	100	166	>250	8.9	9.5	2.8
7e	CH(Me,CH_2OH)	10	100	64	>250	10.0	10.7	1.0
7f	Pr^i	25	100	118	>250	5.5	5.9	4.5
7g	$CH(Et)_2$	10	10	16	131	2.6	2.7	3.8
7h	n Pr	25	100	78	>250	5.1	5.1	4.9
7i	Pe^i	10	50	20	82	4.0	5.5	2.5
7j	Bzl	10	10	19	70	4.1	5.4	2.4

Abbreviations : isopropyl (Pr^i), benzyl (Bzl), n-propyl (n Pr), isopentyl (Pe^i), not relevant (NR).

It appeared that compounds **7f** and **7h** bearing a propylamino chain exhibited the highest safety index (~ 5), so that they could be considered as the most attractive compounds from our *in vitro* evaluation.

Among the *ortho*alkylaminophenols tested, a comparison of the protective effects with the corresponding redox potentials E°' (measured under the experimental conditions reported in reference 13) showed that there was no significant correlation between the observed protection (or toxicity) and the redox potentials. In particular, 2,3-alkylaminophenols **6c**, **6f**, **6g** and **6j** (not oxidizable under our experimental conditions) and 3,4-alkylaminophenols **7a-j** (for which a E'° value of 130-135 mV s.c.e. was found, whatever the nature of the 3-substituted amino chain) exhibited similar neuroprotective activity.

In summary, we have synthesized a series of novel *ortho*alkylaminophenol derivatives as effective neuroprotective agents *in vitro*. Owing to their low safety index (~ 5) compared with those given by another series of 8-amino-1,4-benzoxazine developed in our laboratory[25] (safety index up to 71), the series of 3,4-alkylaminophenols was not considered as promising compounds for therapeutic potential. Nevertheless, they remain potent neuroprotective agents since some of these compounds exhibited an antioxidant activity close to that of standard α-tocopherol.

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17. *Pharmacological evaluation* : HT-22 murine hippocampal cells, a subclone of HT4[18] were maintained (1×10^4 cells/100μL per well) in DMEM/F-12/10% FCS,[19] for 24h in 37°C/5% CO_2. Cellular viability was quantified by measurement of the LDH activity released from damaged cells into the culture supernatant[20] or via the MTT-reduction assay[21] 40-48h after the initial exposure to *L*-HCA.
Neurotoxicity : Drug toxicity after 48h exposure was evaluated relative to mock-treated (0% toxicity) and 50μM menadione-treated cultures (100% toxicity).[22] The MTC and the TC_{50} were estimated using MTT and LDH determinations by linear regression analyses.
Neuroprotection. HT-22 cell cultures in 100μL/well DMEM/F-12/2.5% FCS were preincubated with different concentrations of antioxidant, up to a maximum concentration corresponding to the MTC, for 1h. Cells were then exposed to 2mM[23] L-HCA, in the presence of antioxidant for 48h and neurotoxicity was estimated relative to 2mM *L*-HCA plus 200μM Vitamin-E treated cells (0% toxicity), and 2mM *L*-HCA alone (100% toxicity). The PC_{50} was estimated for both MTT and LDH determinations by linear regression analyses (n = 6 samples / condition).
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