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INHIBITION OF $O_2^{\cdot-}$- AND $HO^{\cdot}$- MEDIATED PROCESSES BY A NEW CLASS OF FREE RADICAL SCAVENGERS: THE N-ACYL DEHYDROALANINES

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N-phenylacetyl dehydroalanines are captodative olefins. They inhibit two processes mediated by superoxide anion ($O_2^{\cdot-}$) in a concentration dependent manner: reduction of NBT to blue formazan and oxidation of epinephrine to adrenochrome. They also inhibit in a dose related way the degradation of deoxyribose produced during either the Fenton reaction or the radiolysis of water, which are the two experimental sources of hydroxyl radical ($HO^{\cdot}$) production. Based on the results obtained with superoxide dismutase, mannitol, thiourea, and uric acid, we postulate that these competitive inhibitory effects suggest a reaction between the dehydroalanine derivatives and the two oxygen derived radicals. Hydroxyl free radical is scavenged more efficiently than superoxide anion. Substitution of the phenyl ring by methoxy groups does not modify significantly the activity. These molecules possess three target active sites which can react with free radicals.

KEY WORDS: Superoxide anion, hydroxyl radical, captodative olefins, N-acyl dehydroalanines, free radical scavenger, AD compounds.

INTRODUCTION

The hypothesis that a substitution by both an electron donor and an electron acceptor group (capto-dative substitution) stabilises a C-centered free radical has been formulated.[1] Olefins having such a capto-dative substituted geminal carbon atom have been shown to react with free radicals. Thus, they have been called radicophiles.[2] They form stabilized free radical adducts which do not undergo typical reactions such as polymerisation or hydrogen abstraction. They rather trap another free radical or dimerize, thus, the free radical-mediated reaction chain terminates. The nature of the capto-dative substitution influences the reactivity of the radicophiles so that molecules with scaled degree of radicophily can be designed.[3]

N-arylacyl-dehydroalanines (AD) form an original class of capto-dative substituted olefins which are identified as AD compounds. In addition to the capto-dative site, they have a methylene group which presents some particular features (Figure 1-a): first, the C–H bond is easy to break, and second, if a free radical abstracts one of the two allylic hydrogen, the radical formed is stabilized by capto-dative substitution (both the carbonyl and the aromatic ring). For this reason, this methylene group may act as a proradical site. Since free radicals react mainly by hydrogen abstraction, electron transfer and by addition to double bonds, AD-compounds may act as

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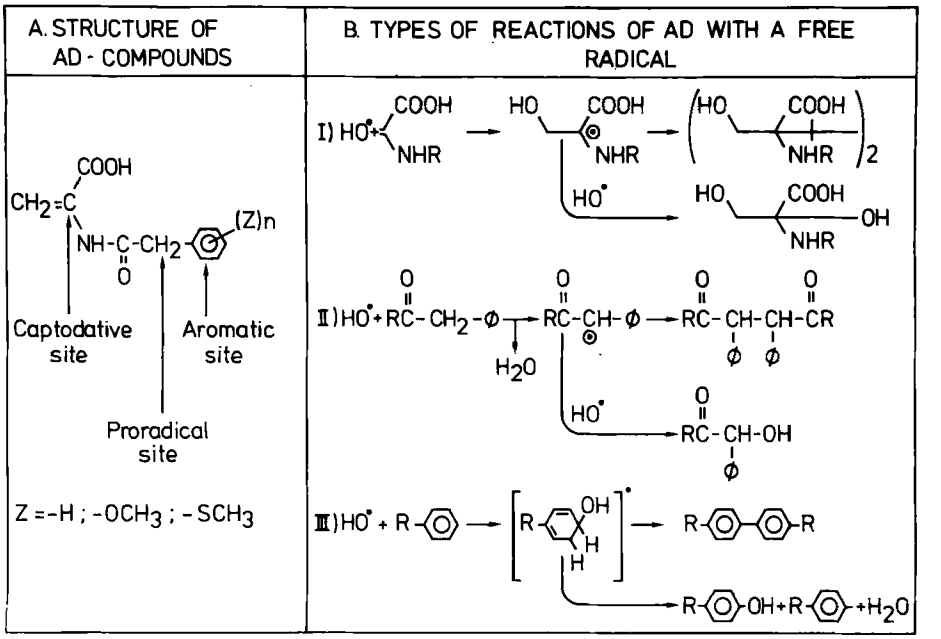

FIGURE 1 Structure of AD-compounds and schematic representation of the reaction patterns between AD and a free radical (e.g. $HO^{\cdot}$).

potential free radical scavengers by offering three different sites. In reaction I (Figure 1-b), the addition of a free radical ($HO^{\cdot}$) to the carbon–carbon double bond forms a capto-dative stabilized free radical adduct which dimerizes or reacts with another free radical; whereas in reaction II, the attack of $HO^{\cdot}$ results in hydrogen abstraction from the methylene group producing another capto-dative stabilized free radical adduct. Moreover, the aromatic ring is a third active site (reaction III), mostly for $HO^{\cdot}$ which adds to produce hydroxylated compounds.[4] Whatever the type of reaction, the propagation of the free radical-mediated chain of reactions will be terminated.

Since the nature of the capto-dative substituents influences the degree of radicophily by modulating the activity at the capto-dative site, the position of substituent on the phenyl moiety of AD-compounds may influence the reactivity of either the proradical site or the aromatic ring and thus may modify the overall activity of the molecule.

The aim of the present work was to test the hypothesis that AD-compounds act as scavengers of oxygen-derived free radicals. The activity of 3 isomeric N-acyldehydroalanine derivatives (Figure 2), the so-called AD-20, AD-27, and AD-5 (ortho-, meta-, and para-methoxyphenyl-acetyl-dehydroalanine respectively), towards $O_2^{\cdot-}$ and $HO^{\cdot}$ was assayed and compared. The oxidation of xanthine by xanthine oxidase and the autoxidation of epinephrine were the model systems used to generate $O_2^{\cdot-}$. The Fenton reaction and water radiolysis were used for $HO^{\cdot}$ production. The effect of AD-5 was compared to that of known free radical scavengers such as superoxide dismutase (SOD), mannitol, glucose, uric acid and thiourea, as a means for evaluating a direct reaction between AD-5 and $O_2^{\cdot-}$ or $HO^{\cdot}$.

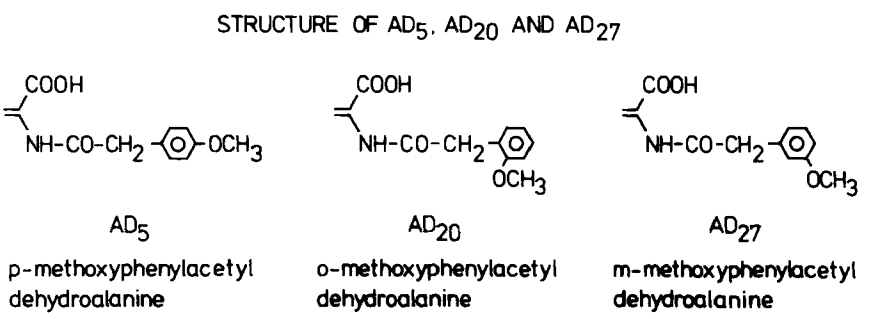

FIGURE 2 Structure of AD-5, AD-20 and AD-27.

MATERIALS AND METHODS

Chemicals

Thymol-free catalase (1 mU/ug); 2-deoxy-D-ribose, superoxide dismutase (3 mU/ug); hypoxanthine, mannitol, uric acid, xanthine oxidase, grade IV (0.18 U/mg); epinephrine, and nitroblue tetrazolium (NBT) were obtained from Sigma Chemicals & Co., (St. Louis, Mi., USA). AD compounds were synthesized in the laboratory of Organic Chemistry (Université Catholique de Louvain) by Prof. Viehe and his coworkers. 2-thiobarbituric acid (TBA) was from Aldrich-Chemie, Steinheim, West Germany. Gum arabic (fine powder), glucose , thiourea and perhydrol were from Merck, Darmstadt, West-Germany. All the other reagents used were of the purest grade available.

Solubilization of AD-compounds

AD-compounds are almost insoluble in water. Therefore, they were either solubilized in dimethylsulfoxide (DMSO) when they were tested in $O_2^{\cdot-}$ -mediated assays or mixed with 0.1 ml of a 2% water suspension of gum arabic for the $HO^{\cdot}$-mediated degradation of deoxyribose (Fenton's assay). For the radiolysis assays, AD-compounds were added as heterogeneous solution in water.

Assays and incubations

All experiments were performed in triplicate. The volume of the incubation was 3 ml. All concentrations are expressed as final concentrations.

Production of superoxide radical. (a) $O_2^{\cdot-}$ was generated by the xanthine–xanthine oxidase model system. Its rate of formation was measured by following spectrophotometrically the reduction of nitroblue tetrazolium (NBT) to blue formazan.[5] The reaction mixtures containing NBT (0.25 mM), hypoxanthine (0.1 mM), EDTA (0.1 mM), 0.05 M sodium carbonate, and concentrations of AD-compounds as indicated in the figures were incubated at 25°C, pH 10.2. The reaction was initiated by adding a sufficient amount of xanthine oxidase to produce a rate of reduction of NBT to blue formazan equivalent to approximately 0.025 Optical Density Units/min at 560 nm. The absorbance variation was converted to nmol of blue formazan produced per min by using the value of $1.5 \times 10^4 \, mM^{-1} \, cm^{-1}$ as a molar extinction coefficient.

(b) O_2^- was also generated during the autoxidation of epinephrine, and measured by adrenochrome assay.[6] The reaction mixture contained EDTA (0.1 mM) in sodium carbonate buffer 0.05 M pH 10.2. After starting the reaction by adding epinephrine (0.33 mM), the adrenochrome formation was followed at 480 nm. It was converted to nmol/min by using a molar extinction coefficient of $4.08 \times 10^3 mM^{-1} cm^{-1}$.

Production of hydroxyl radical. (a) $HO^\cdot$ was produced by the Fenton reaction. It was measured by following the degradation of deoxyribose.[7] The reaction mixture contained: 2-deoxyribose (2 mM), H_2O_2 (0.7 mM) and the iron-EDTA complex $(NH_4)_2Fe(SO_4)_2$ (0.12 mM)/EDTA (0.13 mM) in phosphate saline buffer (PBS) pH 7.4 (24 mM Na_2HPO_4/NAH_2PO_4; 0.15 M NaCl). Incubations were carried out at 37°C under an air atmosphere. After 60 min the reaction was stopped by adding ice cold TCA (2.8%). After vortexing, the mixtures were then centrifuged at 1,000 g for 10 min. Aliquots of the supernatant were mixed with TBA (1% in 0.05 M NaOH) and heated for 15 min in a boiling water bath. After cooling, the extent of the $HO^\cdot$ production was estimated by measuring, at 532 nm, the amount of TBA reacting material produced during the degradation of deoxyribose. The results are expressed as optical density units.

(b) $HO^\cdot$ was also produced by the radiolysis of nitrogen (N_2) saturated water solutions of deoxyribose (2 mM). The samples were purged with N_2 for at least 20 min before irradiation for different periods of time. ^{60}CO was used as a source of gamma-rays with a dose-flux of 13 krads/min. After irradiation, the samples were processed as described above for the iron-mediated degradation of deoxyribose.

RESULTS

Effects of AD-compounds on O_2^- mediated processes

The N-(p-methoxyphenylacetyl)-dehydroalanine AD-5 (used as reference compound) inhibits the O_2^- mediated production of either blue formazan from NBT or adrenochrome from epinephrine (Figure 3). These results show that whatever the redox nature of O_2^- (reducing or oxidizing agent), the AD prevents its reactions. These inhibitory effects were dose-dependent in a mM range with an approximate AD-5 concentration of 4×10^{-3} M to inhibit 50% of the NBT-reduction and 8×10^{-3} M to inhibit 50% of the adrenochrome-formation.

With these values and by taking the rate constants of both NBT and epinephrine with O_2^- from Auclair[8] and Fridovich,[9] respectively, the second order constant for O_2^- trapping by AD-5 was calculated as follows:

$$\begin{aligned} NBT + O_2^- &\rightarrow (NBT/O_2)^\cdot \qquad k_1: 1 \times 10^4 M^{-1} sec^{-1} \\ AD\text{-}5 + O_2^- &\rightarrow (AD\text{-}5/O_2)^\cdot \qquad k_2: ? \end{aligned}$$

When AD-5 inhibits 50% of the NBT reduction, then $v_1 = v_2$ and $k_1 \times (NBT) \times (O_2^-) = k_2 \times (AD\text{-}5) \times (O_2^-)$ (NBT): 0.25 mM
(AD-5): 4 mM

k_2: $k_1 \times (NBT)/(AD\text{-}5) = 1 \times 10^4 \times 0.25/4 = 0.6 \times 10^3$

Similarly for the epinephrine assay (EPI):

$$k_2: k_1 \times (EPI)/(AD\text{-}5) = 1.8 \times 10^4 \times 0.33/8 = 0.7 \times 10^3$$

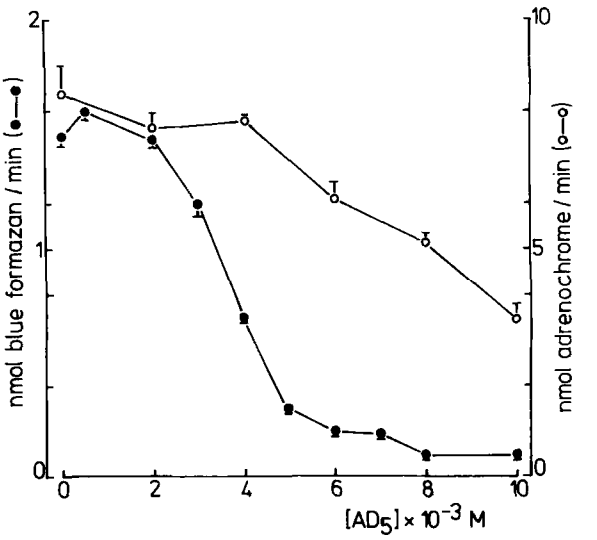

FIGURE 3 Effects of AD-5 on O_2^- mediated NBT reduction and epinephrine autoxidation. NBT reduction (closed circles) was followed at 560 nm whereas epinephrine autoxidation (open circles) was followed at 480 nm. AD-5 was added in 0.02 ml of DMSO. The composition of each mixture and the condition of the incubations are described under Materials and Methods. Each point represents the mean value ± S.D. (N = 3).

Then, the second order constant of the reaction for AD-5 with O_2^- may be estimated at 0.6–0.7 × $10^3 M^{-1} sec^{-1}$

The three isomers (5, 20, and 27) were tested at a concentration of 4 or 8 mM in the NBT- and adrenochrome-assay respectively. All three AD-compounds inhibit both O_2^- mediated processes (Table I), AD-27 being somewhat more active in the NBT-assay than AD-20 or AD-5.

That the redox reactions (reduction of NBT or oxidation of epinephrine) are O_2^--mediated is supported by the fact that more than 95% of inhibition is obtained by the addition of SOD (Table I). Nevertheless it has been described that O_2^- may not

TABLE I
Effects of AD compounds, SOD, mannitol, and CAT on O_2^--mediated redox processes.

	NBT Reduction nmol blue formazan/min	Epinephrine Oxidation nmol adrenochrome/min
Control	1.58 ± 0.03	6.8 ± 0.55
DMSO	1.65 ± 0.02	6.4 ± 0.17
AD-5	0.91 ± 0.02	3.2. ± 0.28
AD-20	0.87 ± 0.03	3.3 ± 0.34
AD-27	0.69 ± 0.04	2.6 ± 0.45
SOD	0.09 ± 0.01	0.2 ± 0.01
Mannitol	1.60 ± 0.02	6.6 ± 0.25
CAT	1.58 ± 0.05	6.8 ± 0.41

The concentrations of AD and mannitol are 4 and 8 mM in the NBT-assay and adrenochrome assay respectively, whereas SOD and CAT were used in both assays at the same concentration, 16.6 and 33.3 ug/ml respectively. Values are mean ± S.D. of 3 separate experiments.

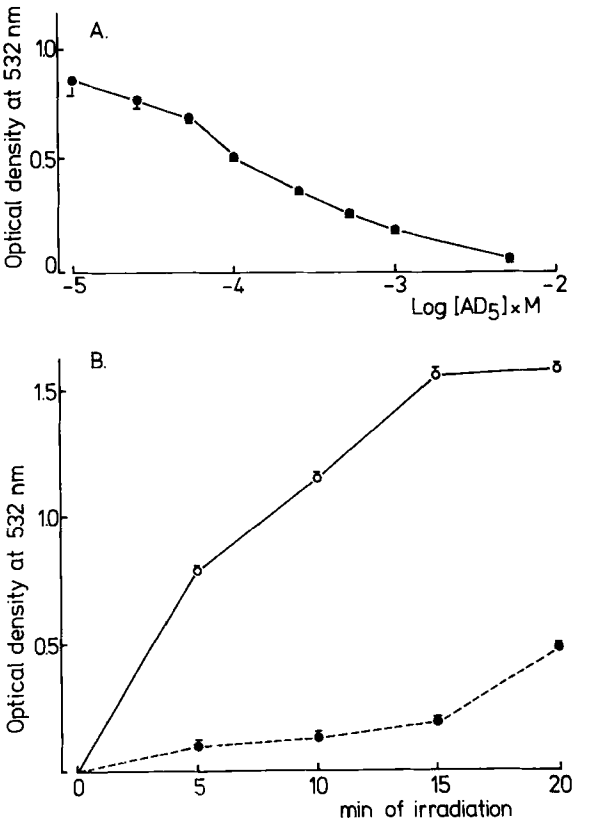

FIGURE 4 Effects of AD-5 on HO˙ mediated degradation of deoxyribose. (A) Dose-dependent effect of AD-5 on the degradation of deoxyribose induced by hydroxyl radical generated by the Fenton's reaction. AD-5 was added in 0.1 ml of a water suspension of gum arabic. (B) Time-dependent accumulation of MDA-like products after deoxyribose degradation by hydroxyl radical generated by the radiolysis of water, in the absence (open circles) and in the presence (closed circles) of AD-5 (1 mM). Each point represents the mean value ± S.D. (N = 3).

be the only product of the xanthine oxidase reaction. Indeed H_2O_2 may also be produced.[10] However in the experimental conditions reported here, the addition of CAT does not inhibit either NBT reduction or epinephrine oxidation showing that H_2O_2 does not play a role in these two reactions. Similarly, mannitol (a well known scavenger of HO˙), was without effect on the two $O_2^{\cdot-}$-mediated processes. These results suggest that HO˙ is not directly involved as an oxidizing agent in the epinephrine process, or in NBT reduction (Table I).

Effects of AD-compounds on HO˙ mediated processes

When HO˙ was produced by the Fenton reaction, AD-5 inhibited the degradation of deoxyribose caused by this radical. The AD-5 concentration required to inhibit 50% of the degradation of deoxyribose is approximately 2×10^{-4} M (Figure 4-A).

To eliminate a chelation of iron by AD, HO˙ was produced by water radiolysis using the gamma rays irradiation of N_2-saturated water solution of deoxyribose. That

TABLE II
Effects of AD compounds and HO· scavengers on HO· mediated degradation of deoxyribose.

	Fenton's Assay	Radiolysis Assay	
	Activity (%)	Activity (%)	$^{k}(HO^{\cdot})^{\S}$
G. Arabic	104 ± 2	N.D.	
AD-5	31 ± 4	56 ± 2	7.6
AD-20	18 ± 3	56 ± 1	—
AD-27	27 ± 3	43 ± 2	—
Mannitol	83 ± 6	80 ± 5	1.0
Glucose	85 ± 3	79 ± 1	1.5
Thiourea	7 ± 4	51 ± 3	3.9
Uric acid	11 ± 2	60 ± 5	7.2

All compounds tested were at 5×10^{-4} M. The activities are expressed as % of the non inhibited process (control activity). Control values for Fenton's reaction and water radiolysis were 1.025 ± 0.097 and 1.013 ± 0.025 Optical Density Units respectively.

Each value is the mean ± S.D. of 3 separated experiments.

N.D. = not determined.

(§) = rate constants were taken from Farhataziz[20] and all values are in $\times 10^{9}\,M^{-1}\,sec^{-1}$

process causes the degradation of sugar to produce a MDA-like substance which reacts with TBA to form a complex which absorbs at 532 nm. The optical density increases with the duration of exposure to ionizing radiation, reaching a plateau after 20 min (Figure 4-B). The addition of AD-5 (1 mM) inhibits by 90% the effect of HO· during the first 15 min and by 70% after 20 min of irradiation. Thus the inhibition of the HO· mediated degradation of deoxyribose by AD-5 is not due to a chelation of iron but rather to a direct scavenging of HO·.

The effects of AD-compounds on the degradation of deoxyribose were evaluated by using the Fenton reaction to generate HO· (Table II). At 5×10^{-4} M all three AD molecules significantly inhibit the degradation of deoxyribose, but some statistical differences were observed. In fact, AD-5 and AD-27 were equally active (70% inhibition), but AD-20 was more active (80% inhibition). Their activity was compared to that of equimolar (5×10^{-4} M) concentration of HO·-scavengers mannitol, glucose, uric acid and thiourea. They were more active than mannitol and glucose but were less active than uric acid and thiourea (Table II).

When deoxyribose was decomposed by HO· generated during the water radiolysis, the three isomers (tested at a concentration of 5×10^{-4} M) inhibited the degradation of deoxyribose with approximately the same efficacity (45%). Similarly, their activity was compared to that of equimolar (5×10^{-4} M) concentration of mannitol, glucose, uric acid and thiourea (Table II). The AD compounds were more active than mannitol and glucose but they had the same effect as thiourea and uric acid.

By using the same procedure to calculate the second order constant for superoxide trapping by AD-5, and a k value of $1.9 \times 10^{9}\,M^{-1}\,sec^{-1}$ for the reaction of HO· with deoxyribose,[12] the rate constant of AD-5 with HO· was estimated at $7.6 \times 10^{9}\,M^{-1}\,sec^{-1}$. That value is in good correlation with the percentage of inhibition observed and the rate constants obtained for mannitol, glucose, thiourea and uric acid, as it can be observed in Table II.

Since thiourea may react with H_2O_2[11] and since uric acid may act as iron chelator[12] (the two reactants of the Fenton's reaction), some of their scavenging activity may be related to these properties. By using the ferrithiocyanate method[13] to assay H_2O_2 it

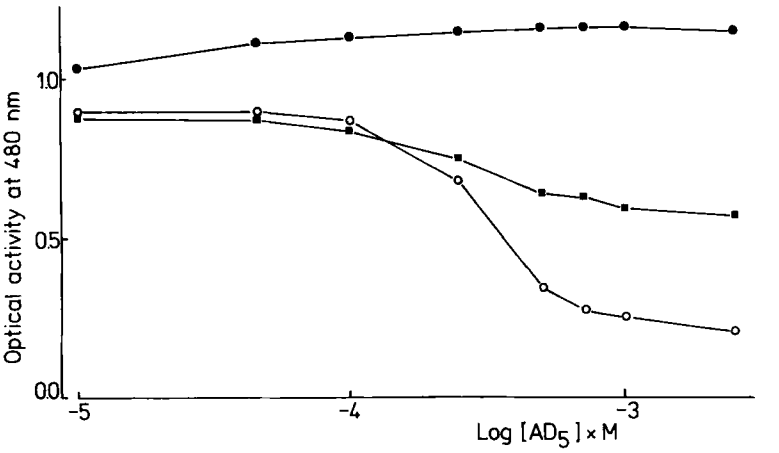

FIGURE 5 Effect of AD-5, thiourea and uric acid on H_2O_2. The solution mixture contained ferrous ions (0.9 mM), potassium thiocyanate (0.11 M), H_2O_2 (0.1 mM), and AD-5 (closed circles), uric acid (closed squares), and thiourea (open circles) at different concentrations (10^{-5} up to 10^{-2} M). The formation of the complex iron-thyocianate was followed at 480 nm. Each value is the mean of two separate experiments.

was shown that AD-5 did not interfere with H_2O_2, whereas uric acid and thiourea did (Figure 5).

DISCUSSION

The validity of the concept of stabilization of free radicals by capto-dative substitution, as proposed by Viehe *et al.*,[2] has been supported by experimental evidences showing that capto-dative olefins are antioxidants and inhibitors of free radical-mediated polymerisation.[14-16]

N-(methoxyphenyl-acetyl)-dehydroalanine derivatives (indexed as AD-compounds), are capto-dative olefins which furthermore have a capto-dative proradical site and an aromatic ring. They have been tested for their scavenging activity towards O_2-derived free radicals, such as $O_2^{\cdot-}$ and $HO^{\cdot}$. The experimental evidences reported here demonstrate that AD compounds inhibit $O_2^{\cdot-}$ and $HO^{\cdot}$ mediated process most likely via a direct reaction with the free radical species. As shown for one isomer (AD-5) these effects are concentration dependent. As with their effectiveness towards $O_2^{\cdot-}$ and $HO^{\cdot}$, the concentration to inhibit 50% of the redox processes are in the order of 4–8 $\times$ 10^{-3} M and 2–4 $\times$ 10^{-4} M respectively. That difference may be explained by the difference in reactivity of both oxygen radicals, $HO^{\cdot}$ being more active than $O_2^{\cdot-}$. The second order constant for the reaction of $O_2^{\cdot-}$ with AD is 0.6 $\times$ 10^3 M^{-1} sec^{-1}, whereas the constant for $HO^{\cdot}$ with AD is 7.6 $\times$ 10^9 M^{-1} sec^{-1}.

Even though there are some minor differences between the effects of the three isomers tested with regard to their inhibitory effect towards $O_2^{\cdot-}$ mediated processes, these differences should not be overemphasized. Indeed they are only of the order of 10%. However concerning their inhibitory effects towards $HO^{\cdot}$, it appears that a methoxy group in ortho position may influence positively the activity of the molecule at the proradical site level by combining the capto-dative stabilization (a ther-

modynamic effect) with a steric protection and/or a solvation effect leading to a kinetic stabilization (Figure 1).

As to the question of which of the 3 reactive sites of the AD compounds react with $O_2^{\cdot-}$, the results presented here do not allow to give a final answer. However it may be speculated that the most probable reaction between AD and $O_2^{\cdot-}$ (or $HO_2^{\cdot}$) may be either the transfer of an electron or the abstraction of an hydrogen atom to give a capto-dative carbon-centered radical adduct. In both cases, the free radical scavenging activity of AD compounds towards $O_2^{\cdot-}$ may be at the capto-dative site level. Further work is in progress in order to identify the nature of the radical adduct and the mechanism of this reaction.

In relation with hydroxyl radical, as it reacts by hydrogen abstraction or by addition to the double bond of either the olefin or the aromatic ring, it is at present difficult to choose among the three possibilities. Further work is in progress to solve that problem.

In both Fenton reaction and water radiolysis experiments, AD-compounds are more active than mannitol and glucose. Thiourea and uric acid have the same level of activity than the AD-compounds when $HO^{\cdot}$ is produced by water radiolysis, but they have a higher effect when that radical is the product of a Fenton reaction (Table II). This discrepancy could be explained by the fact that thiourea reacts with hydrogen peroxide[11] and uric acid chelates the iron,[12] whereas AD compounds do not. Thus, when $HO^{\cdot}$ was generated in the absence of both iron and H_2O_2 (water radiolysis), thiourea and uric acid were less active to inhibit the degradation of deoxyribose (Table II). Since AD compounds do not react either with H_2O_2 (Figure 5) or with TBA or MDA (data not shown). their inhibitory effect on $HO^{\cdot}$-mediated degradation of deoxyribose suggest a direct reaction between AD and $HO^{\cdot}$.

The central role of $O_2^{\cdot-}$ in both NBT-and epinephrine-assays is confirmed by the experiment showing that SOD totally inhibits both reactions (Table I). Similarly the use of thiourea and uric acid, two $HO^{\cdot}$ scavengers, unable to react with $O_2^{\cdot-}$, confirm the role of this radical in the test used (Table II).

The hypothesis concerning the scavenging ability of AD-compounds towards oxygen-derived free radicals is supported experimentally by the results reported here. Since the generation of oxygen-derived free radicals in tissues may lead to biological damages,[17–19] the trapping of these oxygen radicals by AD-compounds could be of biological interest in cell protection against oxidative injuries.

List of abreviations utilized

(AD: arylacetyldehydroalanine; MDA: malondialdehyde; TBA: 2-thiobarbituric acid; CAT: catalase; SOD: superoxide dismutase; NBT: nitroblue tetrazolium; TCA: trichloracetic acid; DMSO dimethylsulfoxide; EDTA: ethylendiamine-tetraacetic acid; PBS: buffer phosphate saline.

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