Pergamon

Bioorganic & Medicinal Chemistry Letters, Vol. 6, No. 19, pp. 2329-2332, 1996

0960-894X/96 $15.00 + 0.00

PII: S0960-894X(96)00422-2

OXIDATION OF RETINYL ACETATE AND ANALOGUES BY NITRIC OXIDE AND NITROGEN DIOXIDE

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Abstract: Nitric oxide did not react directly with retinyl acetate but, in the presence of an initiator, a series of nitroxide radicals was produced and observed by EPR spectroscopy. Product analyses showed that rapid oxidative degradation of retinyl acetate gave a series of carbonyl compounds. NO operating in concert with a self-derived initiator such as NO_2 on unsaturated lipids could account for the cytotoxicity of this molecule.

Following the identification of the endothelium-derived relaxing factor (EDRF) as NO[1,2], major advances have been made in the last decade towards an understanding of the part played by NO in smooth muscle relaxation[3]. It rapidly became apparent that the L-arginine-NO biosynthetic pathway was involved in a multiplicity of biological functions[4] including its potent cytotoxicity under a variety of physiological conditions. For example, several groups have shown that cytotoxic-activated macrophages (CAMs) produce NO as part of the immune response[5,6]. NO contains an unpaired electron and hence it is a free radical, but, on the scale of radical reactivity it lies at the slow rate extremum for most reaction partners. Thus, its radical nature alone is not sufficient to explain its cytotoxic action which is consequently a matter of current research. NO reacts with oxygen to give nitrogen dioxide, a more reactive molecule:

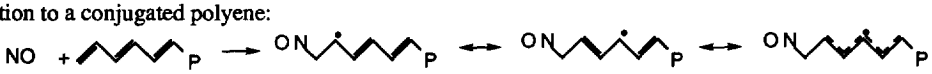
$$2NO + O_2 \rightarrow 2NO_2 \quad (1)$$

Nevertheless, because the half-life of NO_2 production is several hours in aqueous solution at the concentrations that exist in cells[7], process (1) seems unlikely to account for NO cytotoxicity. A more plausible route invokes the reaction of NO with superoxide to give peroxynitrite ($ONOO^-$) which decomposes with production of biologically destructive hydroxyl radicals[8,9]:

$$ONOO^- + H^+ \rightarrow ONOOH \quad (2)$$

$$ONOOH \rightarrow NO_2 + HO^\bullet \quad (3)$$

Pure NO does not react with alkenes in solution, in the absence of a radical initiator; probably owing to the initially formed nitrosoalkyl radicals being comparatively unstabilised. It seemed possible, however, that direct addition of NO might be favoured if the adduct radical was stabilised by resonance delocalisation as would occur on addition to a conjugated polyene:

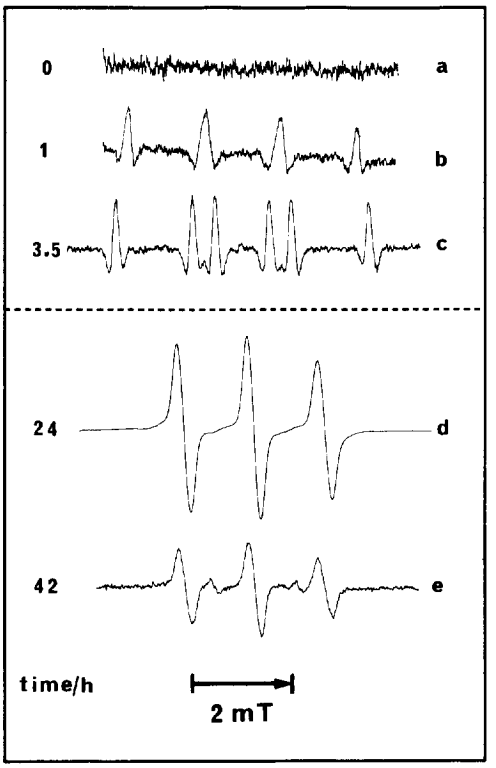


(4)

In view of this, a suitable substrate must contain two or more conjugated double bonds and such materials, e. g. retinol and β-carotene, are widely distributed *in vivo*. A recent report revealing that NO reacts with β-carotene to produce a series of nitroxides[10] lends encouragement to this idea.

To explore this option further we examined the reaction of NO with retinyl acetate (**1**), β-ionyl acetate and allyl acetate, a sequence of molecules in which the extent of π-bond conjugation steadily decreases. Pure NO was made from solutions of ascorbic acid and sodium nitrite that had been thoroughly degassed with N_2 before use and passed into a degassed solution of retinyl acetate (0.13 M) in benzene. Experiments were carried out in a 5 mm dia. tube directly in the cavity of an EPR spectrometer, and also on a larger scale in a flask outside the cavity from which samples, removed periodically by a syringe, were analysed and examined by EPR

spectroscopy. During the first 60 min. of continuous NO passage into the solution no EPR signals were detected (Figure, a). Over the next 2.5 h, first a 4 line signal appeared (Figure, b), this passed through a maximum and was gradually replaced by a 6 line spectrum (Figure, c). The 6 line signal itself decayed over the next few h and was replaced by a strong 1:1:1 triplet (Figure, d). By 42 h the 1:1:1 triplet intensity had diminished and a new triplet with a narrower hyperfine splitting (hfs) was beginning to appear (Figure, e).

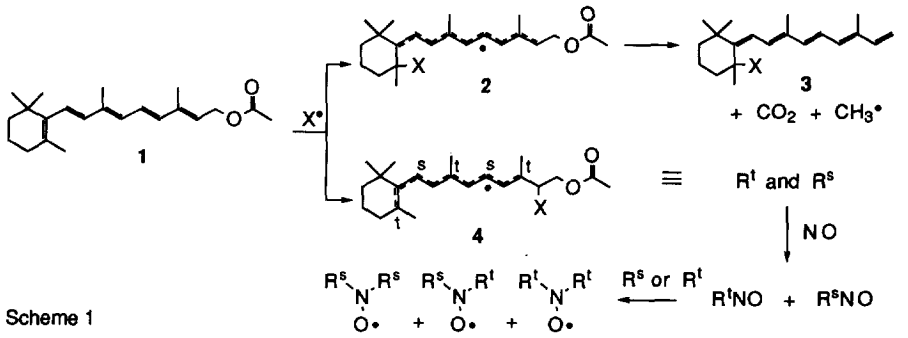


9.1 GHz EPR spectra of radicals generated from **1** in benzene solution by passing NO gas at 293 K. Upper box: second derivative spectra. Lower box: first derivative spectra.

Spectrum:	a	b[11]	c	d	e
React. time/h[Gain]:	0[2x10^5]	1[4x10^5]	3.5[3x10^5]	24[4x10^4]	42[2x10^5]

We interpret these spectral changes as follows. Pure NO did not react with retinyl acetate and hence no signals were observed during the first hour. The subsequent development of spectra from nitroxide radicals (aminoxyls) results from the formation of an initiator radical (X$^\bullet$) which did add to **1** producing a delocalised radical that combined with NO to give nitroso compounds. The EPR hfs of the first species (Figure, b)[11] and the second species (Figure, c), determined by computer simulations, were: a(N) = 15.1, a(1H) = 14.3 and a(N) = 14.6, a(1H) = 18.8 G respectively. These parameters indicated that both species were dialkyl nitroxides containing one tertiary and one secondary alkyl group i.e. $R^1R^2R^3CN(O^\bullet)CHR^4R^5$. The magnitude of the hfs from the β-hydrogen in such radicals depends strongly on the conformation about the N—C_β bond and so the observed difference in the a(1H) values points to the fact that the two species have different substituents R^4 and R^5. The nitrogen hfs of the strong triplet (Figure, d) (13.3 G) indicated formation of di-*t*-alkyl nitroxides, the relatively large linewidth suggested that several similar radicals of this type overlapped to give the observed signal. The final weak triplet had a(N) = 7.7 G which is indicative of a *t*-alkylacylnitroxide, $R^tN(O^\bullet)C(O)R$.

A rationale for the process, analogous to previous proposed mechanisms for reaction of NO with dienes[12], is outlined in Scheme 1. The identity of initiator radical X• is uncertain but it is probably NO_2 resulting



Scheme 1

from NO/O_2 interaction, equ. (1). Although air was excluded from the system, it is concievable that, after 1 h, minute quantities of O_2 had built up either by degassing from internal surfaces or by seepage. This conclusion was supported by the observation that when small amounts of air were deliberately introduced into the system via a syringe, leading to NO_2 formation *via* reaction (1), a similar sequence of spectra resulted without an induction period. Under these conditions the strong triplet (Figure, d) dominated from an earlier stage. Addition of NO_2 will occur preferentially at the two ends of the conjugated system of **1** because this leads to adduct radicals **2** and **4** which have maximum resonance stabilisation. Product analyses (see below) suggested that **2** underwent β-scission to give a methyl radical together with the conjugated polyene **3** and CO_2. Acetoxy group migration in related β-acetoxyalkyl radicals is well documented and whether the mechanism is elimination followed by re-addition, or concerted, has been vigourously debated[13]. The resonance stabilisation of radical **2** would disfavour acetoxy migration and therefore elimination may predominate because this produces a conjugated polyene, **3**. Fragmentation of the released acetoxy radical to methyl and CO_2 is known to be rapid[14]. The spin density of the delocalised unpaired electron in radical **4** has maxima at secondary and tertiary sites marked *s* and *t* in structure **4**. Thus, combination of NO with **4** will lead to 5 different nitroso compounds, 2 secondary and 3 tertiary. Both types of nitroso compound will pick up a second radical **4** at either a *s* or *t* site producing a mixture of three sorts of dialkylnitroxides (Scheme 1). The di-*s*-nitroxides ($R^sN(O^•)R^s$) will be too short-lived for EPR detection because they decay rapidly by disproportionation. The *s,t*-nitroxides will be longer lived and two of these are well enough resolved for detection (Figure, b, c). The di-*t*-alkylnitroxides have much longer lifetimes because disproportionation is not possible and therefore they eventually dominate. Clearly, several different di-*t*-alkyl nitroxides can be formed and they overlap to give the rather broad lines of Figure, d. Further oxidation of **1**, **3** and the nitroxides gave carbonyl compounds from which the final carbonyl nitroxide (Figure, e) was derived.

The products of the reaction were examined at each stage by GC-MS (EI and CI) which confirmed the absence of reaction in the first 1 h and showed the subsequent steady emergence of (i) a string of alkyl aromatics (xylenes, diisopropylbenzenes, other polyalkylbenzenes) and (ii) a series of carbonyl compounds (β-ionylidene acetaldehyde, β-ionone, β-cyclocitral, 2,2,6-trimethylcyclohexanone, dihydroactinidiolide and others). The alkylbenzenes probably resulted from addition of the methyl radicals to the solvent giving, after oxidation, toluene which experienced further addition reactions to produce xylenes; attack at the benzylic hydrogens would eventually afford other polyalkylbenzenes. The carbonyl compounds are similar to those identified from the oxidation of other retinoids and carotenoids[15,16]. In this context, adduct radicals **2** and **4** must acquire oxygen at

the *s* and *t* sites and undergo oxidative scission of each of the double bonds, via an established mechanism[15,16]. The entire sequence of events culminates in the observed products. In the later stages of the oxidation, the nitroxides will themselves undergo oxidative degradation affording the same carbonyl compounds.

In similar reactions with NO, both allyl acetate and β-ionyl acetate showed induction times of ca. 0.5 h before development of EPR signals. The only identifiable spectrum from the former substrate was that of an alkylacylnitroxide and β-ionyl acetate showed only broad unidentifiable signals. These results are in accord with the inactivity of any of these substrates towards pure NO. There are no tertiary sites in allyl acetate hence dialkyl nitroxides derived in a manner analogous to the Scheme would be too short-lived for detection; in agreement with the lack of such EPR signals. Product analysis showed similar series of alkylbenzenes which, for both compounds, were consistent with initiation and subsequent degradation steps akin to those depicted in the Scheme.

The experimental observations support the conclusion that NO does not add directly to conjugated systems of double bonds to yield delocalised radicals as proposed in equ. (4). However, carbon-centred radicals formed from addition of initiator radicals (probably NO_2) to polyunsaturated lipids rapidly combine with NO to give nitroso compounds which undergo oxidative degradation. The relative contribution of this process to the cytotoxicity of NO will depend critically on the rate of NO conversion to NO_2, equ. (1). Although this is slow in aqueous media, the present study points towards this pathway being either enhanced in the lipid phase or that other initiator radicals may act in conjunction with NO causing an abnormal turnover of unsaturated lipids to carbonyl compounds.

Acknowledgements: We thank the EPSRC and GlaxoWellcome for financial support of this research.

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11 Note that the two inner lines in Figure b are broader than the outer lines due to overlap of spectral components; in Figure c the inner lines are fully resolved.
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(*Received in Belgium* 9 *July* 1996; *accepted* 6 *September* 1996)