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Medium-dependent Competitive Pathways in the Reactions of Polyunsaturated Fatty Acids with Nitric Oxide in the Presence of Oxygen. Structural Characterisation of Nitration Products and a Theoretical Insight

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Abstract

Reactions of methyl arachidonate and ethyl linoleate with NO in the presence of O_2 gave complex mixtures of products *via* interaction with NO_2 and related nitrogen oxides. In cyclohexane, the reaction afforded chiefly nitroalkene and nitronitrate adducts, characterised by spectroscopic techniques. In 0.1 M phosphate buffer, pH 7.4, a different pattern of products was formed, comprising mainly conjugated hydroperoxide derivatives. Quantum mechanical calculations on a model 1,4-diene system suggested that medium-dependent changes in product distribution can be ascribed in part to solvation effects on the NO_2-induced addition/H-atom abstraction competition, but reflect also a complex interplay of solvent-sensitive mechanisms.

Keywords: Lipids, nitration, nitrogen oxides, theoretical studies

1. Introduction

Originally aroused by the toxicological relevance to the noxious effects of cigarette smoke and severe atmospheric pollution, interest in the reactions of nitrogen oxides with polyunsaturated fatty acids has recently been revived by the discovery of the dichotomous role of

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endogenously produced nitrogen monoxide (NO, commonly referred to as nitric oxide [1,2]) as either modulator or amplifier of peroxidative membrane damage and tissue injury in a variety of pathophysiological conditions [3]. Studies of the effects of NO on lipid peroxidation have underscored the ability of this radical to intercept chain-propagating peroxyl and alkoxyl radicals, as well as other lipid radicals, to give a variety of nitrite and nitrate ester derivatives [4-6].

Though relatively inert *per se*, NO may be oxidised by O_2 and reactive oxygen species to a range of nitrogen oxides, including chiefly NO_2 [7,8] and peroxynitrite [9,10], which display pronounced free radical reactivity toward alkenes [11-19] and are capable of initiating lipid peroxidation [5,20]. The diversity of mechanistic options featured by this chemistry emerged in a series of fundamental studies of the reactions of NO_2 with methyl linoleate and other lipids [21-24]. These highlighted a concentration- and oxygen-dependent competition between homolytic addition and hydrogen abstraction processes, the latter leading to allylic nitro and nitrite derivatives and/or hydroperoxides. Nitration of polyunsaturated fatty acids was also reported in reactions with NO/O_2 [25], peroxynitrite and other NO-derived nitrogen oxides [26,27], most of the latter studies being performed with low concentrations of nitrogen oxides and/or under physiologically relevant conditions. Whether the same patterns of reactivity also dominate the behaviour of polyunsaturated fatty acids in the presence of large concentrations of NO in the presence of O_2 has received comparatively less attention, despite the potential synthetic interest of these reactions in lipid chemistry. In the present study we surveyed the reactions of polyunsaturated fatty acids and esters with NO in air-equilibrated media representing opposed extremes for what concerns the substrate solvation and polarity. Scope of the work was to provide a detailed spectral characterisation of the resulting nitration products as an improved basis for their detection in in vitro and in vivo systems, and to explore the potential of quantum mechanical approaches to discriminate among the possible mechanistic options.

2. Results and Discussion

2.1 Reactions of polyunsaturated fatty acids with NO in cyclohexane

Exposure of arachidonic acid to NO at 25 °C resulted in a smooth reaction whose extent depended on several parameters, including NO concentration, efficiency of oxygenation, and nature of the medium. In a rigorously oxygen-free atmosphere little or no substrate was consumed, consistent with the poor reactivity of NO towards alkenes and related substrates in the absence of a radical initiator [18,28]. From a reaction mixture in aerated cyclohexane, a main homogeneous fraction positive to Griess reagent [29] could be isolated, which displayed quite strong bands in the FT-IR spectrum at 1656 and 1564 cm^{-1}, and a moderately intense band at 1377 cm^{-1}, suggesting incorporation of NO. Unfortunately, all attempts to isolate some

representative products in pure form were defeated because of the exceeding complexity of the mixture and the unfavourable chromatographic properties of the acid derivatives.

To gain a deeper insight into the nature of the NO-lipid adducts, purified NO was slowly bubbled through a solution of $1x10^{-3}$ M methyl arachidonate in cyclohexane. After a few minutes, a complex pattern of Griess-positive products was obtained, from which a chromatographically homogenous fraction (about 15% w/w, 20% yield based on reacted substrate) could be separated by preparative TLC. The UV spectrum lacked well defined chromophoric features, whereas the FT-IR spectrum exhibited intense bands at 1649, 1559, 1523, and 1335 cm^{-1}, but no absorption in the OH-stretching region, suggesting nitrate and nitro groups, the latter linked to both sp^2 and sp^3 carbons. Close inspection of the ^{1}H-NMR and ^{13}C-NMR spectra, aided by 2D correlation experiments, revealed an intimate mixture of structurally related species arising apparently by modification of the tetraene moiety of methyl arachidonate. Peculiar features of the spectra included: *a)* a set of three multiplets centred at δ 2.55, 2.95 and 3.35 (ca. 0.5H, 1.5H and 1H in that order, with respect to the area of the 3H methoxyl resonance at δ 3.66 taken as internal reference), correlating with clusters of signals in the ^{13}C-NMR at δ 23-34; *b)* a multiplet at δ 7.1 (ca. 0.5H), typical of protons on *E*-nitroalkenes [30], correlating with carbons resonating at about δ 140; and *c)* a broad multiplet centred at δ 5.4 (ca. 7.5H), which correlated with two distinct clusters of ^{13}C signals in the ranges between δ 78-88 and δ 122-142, denoting both sp^3 methines, bearing nitrate and nitro groups, and olefinic CH groups. The multiplets at δ 2.55 and 3.35 were ascribed to allylic and doubly allylic methylene protons deshielded by adjacent nitro groups. On this basis, it was concluded that the isolated fraction consisted of an intimate mixture of regio- and stereoisomeric nitronitrate adducts (e.g., **I** and **II**) and *E*-nitroalkenes (e.g. **III** and **IV**) (Figure 1).

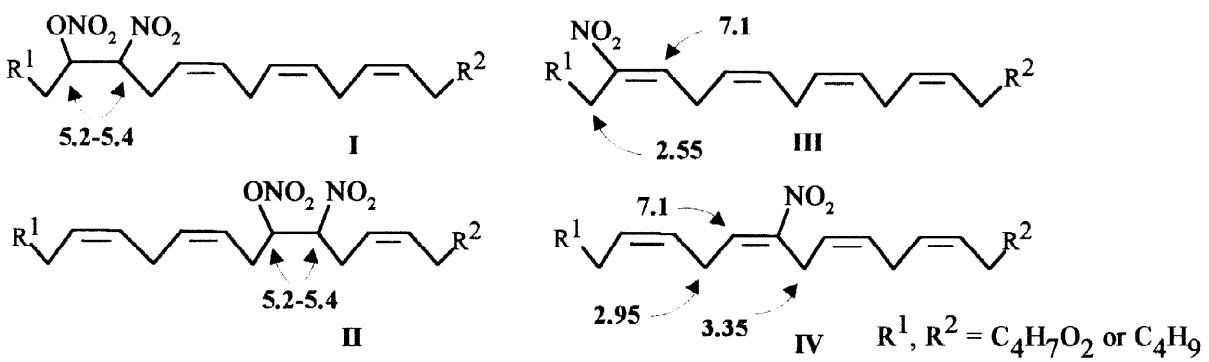


Figure 1. Representative structures of NO-adducts isolated from reaction of methyl arachidonate with NO in cyclohexane. Highlighted proton resonances have been assigned through 2D correlation experiments.

The integrated area of the multiplet at δ 7.1 suggested that nitroalkene products of the type **III**

and **IV** accounted for about 50% of the fraction. Fairly similar NMR spectra were obtained from different preparations, indicating reproducible reactions. Moreover, no significant changes were observed on repeated fractionation of the mixture on silica gel plates. Distinct fragmentation patterns could be discerned in the EI-MS spectrum of the isolated fraction, most of which could be interpreted in terms of cleavage at both sides of a nitro-substituted tetraene moiety (Figure 2).

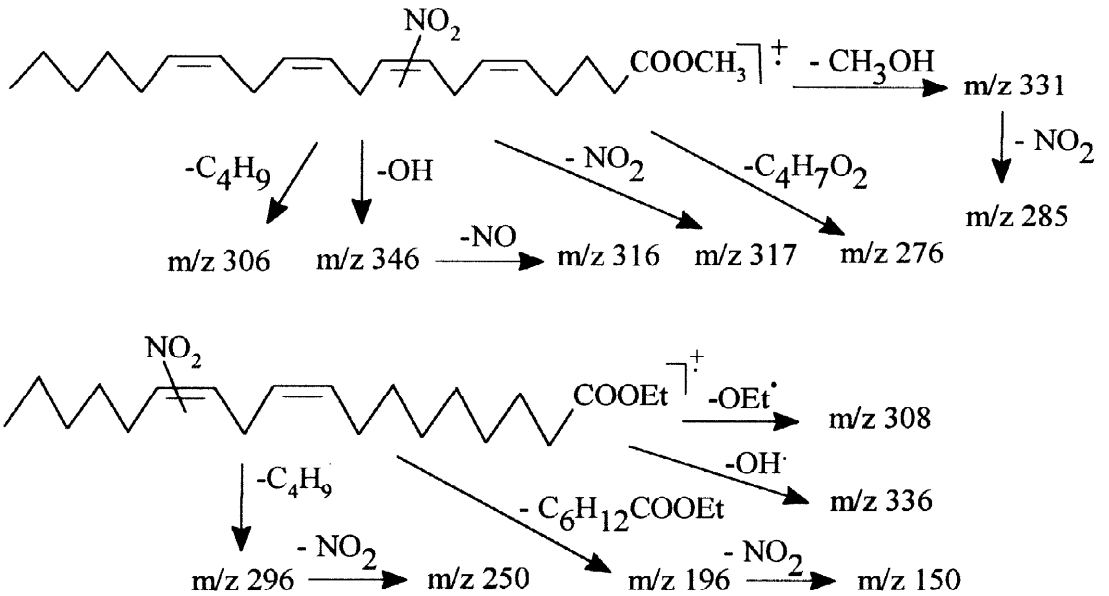


Figure 2. EI-MS fragmentation patterns of adducts of NO to methyl arachidonate and ethyl linoleate.

The lack of diagnostic ^{1}H- and ^{13}C-NMR resonances and IR bands, the particular reaction conditions and the isolation procedure adopted, involving repeated TLC on silica, argue against the presence of detectable amounts of hydroxynitro compounds and unstable nitrite esters in the isolated fraction. Apparently, the remainder of the reaction mixture consisted of polynitro derivatives which escaped fractionation and detailed characterisation. GC-MS techniques proved likewise of little value because of the exceeding complexity of the product mixtures and their tendency to eliminate. No significant formation of hydroperoxides and other H-atom abstraction products or conjugated dienes could be detected in reaction mixtures in cyclohexane.

A better insight into the reaction of NO with unsaturated lipids in air-saturated cyclohexane was gained using as substrate ethyl linoleate, whose features were compatible with the formation of a lower number of isomeric adducts more amenable to structural investigation. Following exposure to NO, mixtures of isomeric nitroolefin and nitronitrate derivatives (ca. 30-40% w/w) could likewise be obtained [25], which resisted all attempts at chromatographic fractionation. GC-MS analysis revealed four main peaks A-D eluted at 32.6, 32.7, 32.8 and 32.9 min (relative abundances ca. 60, 100, 40 and 80% in that order). These featured main fragmentation peaks at m/z 336, 308, 277, 196, 150 (A); 336, 296, 250 (B); 336, 308, 277, 196, 150 (C); and 336, 296,

250 (D), suggesting four distinct sets of intimately related products (Figure 2). The structural properties of the isomeric nitroalkene and nitronitrate adducts produced by reaction of NO with polyunsaturated fatty acids in the presence of oxygen were briefly explored by molecular mechanics calculations [31] (MM+ force field), using the *E*-nitroalkene and nitronitrate derivatives **1-4** as computationally more tractable models. The results indicated that, among the nitroalkene derivatives, *E* isomers are significantly less energetic than *Z* isomers (Table 1). This is consistent with the observed detection of *E* isomers by ^{1}H-NMR and with the known tendency of nitroalcohols and related species to eliminate to give chiefly (*E*)-nitroalkenes [30]. Interestingly, geometry optimisation of the various regio- and stereoisomers investigated gave structures in which the nitro group is considerably twisted out of the plane of the double bond, due evidently to significant steric encumbrance compared to nitroethylene. Less pronounced differences were found in the relative energies of the isomeric nitronitrate derivatives **3** and **4**.

CH_3-CNO_2=CH-CH_2-CH=CH-CH_3 **1**

CH_3-CH=CNO_2-CH_2-CH=CH-CH_3 **2**

CH_3-CH(NO_2)-CH(ONO_2)-CH_2-CH=CH-CH_3 **3**

CH_3-CH(ONO_2)-CH(NO_2)-CH_2-CH=CH-CH_3 **4**

Table 1.
Relative energies and geometric parameters calculated at the MM+ level for compounds **1-4**.

Compd.	Stereochemistry	Energy[a] (kJ mol^{-1})	Dihedral angle (degrees) CCNO	NCCO
1a	*E,Z*	0.0	13	
1b	*Z,Z*	4.2	43	
2a	*E,Z*	3.8	44	
2b	*Z,Z*	8.4	54	
3a	*R,R; Z*	2.1		63
3b	*R,S; Z*	2.1		179
4a	*R,R; Z*	0.8		63
4b	*R,S; Z*	0.0		180

[a]Relative to the energy of **1a** (for compounds 1 and 2) and of **4b** (for compounds **3** and **4**).

2.2 Reactions of polyunsaturated fatty esters with NO in aqueous buffer

When NO gas was slowly bubbled through a vigorously stirred emulsion of methyl arachidonate in air-equilibrated 0.1 M phosphate buffer, pH 7.4, a smooth reaction occurred leading mainly to Griess-negative UV-absorbing species (λ_{max} = 235 nm) with chromatographic and spectroscopic properties suggestive of conjugated diene derivatives. Under such conditions, formation of Griess-positive adducts was markedly decreased. Likewise, reaction of ethyl linoleate with NO in 0.1 M phosphate buffer at pH 7.4 afforded, after reduction with sodium borohydride, a main UV-absorbing fraction consisting of a mixture of isomeric ethyl hydroxyoctadecadienoates, possibly derived from the corresponding hydroperoxides.

2.3 Mechanistic issues and theoretical approach

Two interesting aspects of the reactivity of nitrogen oxides with polyunsaturated fatty acids emerged from the present study: the significant formation of 1,2-nitronitrate derivatives, usually reported as minor products in the reactions of NO or NO_2 with 1,2-disubstituted olefins [16,17]; and the prevalent generation of allylic H-atom abstraction products in aqueous medium even in the presence of high concentrations of NO_2 and other nitrogen oxides, *i.e.* under conditions expected to favour addition over H-atom abstraction routes [21].

To dissect the mechanisms underlying reactions of nitrogen oxides with polyunsaturated lipids, it is necessary to contend with several distinct possibilities, mutually not exclusive, which may be difficult to distinguish experimentally. An additional complication, which is missing in most of the previous studies, arises from the use of NO in the presence of oxygen, entailing involvement of a range of nitrogen oxides. Under conditions of slow, constant supply of NO in air-equilibrated media, the main nitrogenous species would conceivably include, besides NO_2, some N_2O_3, due to the trapping of NO_2 by NO, but expectedly little N_2O_4, since the excess of NO would prevent the rapid increase of NO_2 levels required for dimerisation (see discussion in [32]). Interaction of the unsaturated fatty acids with NO_2 or a related species would thus initiate divergent free radical pathways schematically illustrated in Figure 3.

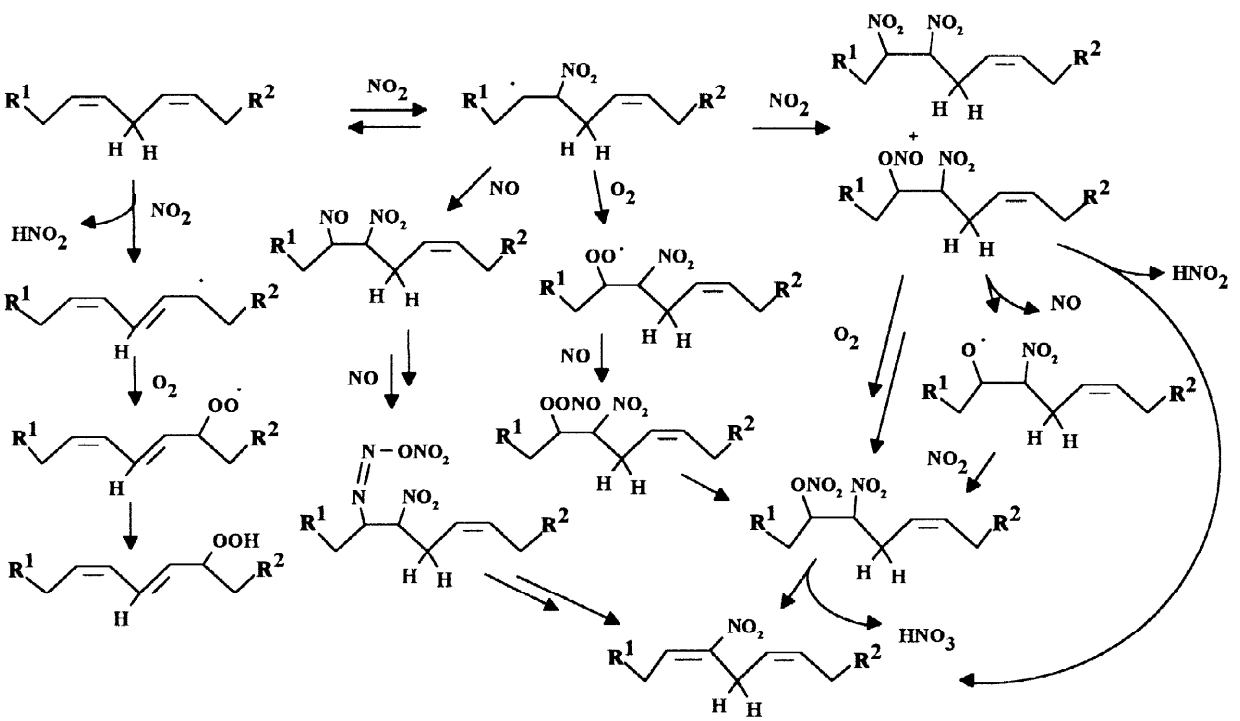
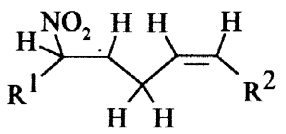
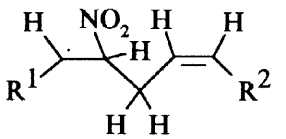


Figure 3. Possible origin of nitronitrates, nitroalkenes and allylic H-atom abstraction products by reaction of unsaturated fatty acids with the NO/O_2 system. For simplicity, only representative structures are shown.

In cyclohexane, the initial event is likely to be addition of NO_2 or a related nitrogen oxide to

the olefinic double bond, leading to β-nitroalkyl radical intermediates. Given the reversibility of this step [13,21,22], it can be argued that the regiochemistry of nitration is under thermodynamic control and can be dictated by the relative stability of the isomeric nitroalkyl radicals. To address this issue, the relative energies of representative radicals **5a** and **6a** and, for comparative purposes, of their models **5b-c** and **6b-c** were examined by molecular mechanical (MM+) and quantum mechanical approaches (Table 2). In the latter case we used the Gaussian 98 package [33] and the B3LYP density functional, which is reliable for the study of radical species [34,35] when coupled to basis sets of at least polarised split valence quality (here 6-31G*).





5a $R^1=C_5H_{11}$, $R^2=C_8H_{15}O_2$ **6a** $R^1=C_5H_{11}$, $R^2=C_8H_{15}O_2$

5b $R^1=CH_3$, $R^2=CH_3$ **6b** $R^1=CH_3$, $R^2=CH_3$

5c $R^1=H$, $R^2=H$ **6c** $R^1=H$, $R^2=H$

Table 2.
Relative energies (kJ mol^{-1}) of nitroalkyl radicals **5** and **6** calculated at the MM+ and B3LYP/6-31G* level.

Compound[a]	MM+	B3LYP
5a	0.0	--
6a	1.7	--
5b	0.0	0.00
6b	3.3	2.26
5c	0.0	0.00
6c	4.2	2.59

[a]Geometries obtained at the MM+ level.

Both molecular mechanical and quantum mechanical calculations indicated that, for the model systems, radicals derived from addition of NO_2 to the terminal position of the polyene system (**5**) are more stable than those with nitro groups on the inner positions (**6**), energy differences increasing with decreasing chain length. Putting aside the **5c/6c** couple, in which addition is evidently favoured at the terminal position because of the generation of a more stable secondary radical, these data suggest that steric factors may be important in determining the relative energies of nitro radicals, and predict a poor regioselectivity in the addition of NO_2 or a related species to fatty acid double bonds. This would be in accord with the nearly statistical distribution of regioisomeric nitroalkene products observed in the present study, although the limited fraction of products examined precludes any definitive conclusion about this point.

Just generated, the nitroalkyl radical can partition among three distinct coupling pathways with

NO, NO_2 or O_2. Reaction with NO would expectedly give mainly nitroalkenes via nitronitroso derivatives [28]. Coupling with NO_2 may occur via both the oxygen and nitrogen centres, leading to 1,2-nitronitrite and 1,2-dinitro adducts, respectively. In the case of polyunsaturated fatty acids the latter route is expectedly favoured, based on literature data for substituted alkenes [17]. Finally, the nitroalkyl radical could be intercepted by oxygen to give a nitroperoxyl radical [12] which would then couple with NO at diffusion-limited rate ($k=2x10^9$-$11x10^9$ M^{-1} s^{-1}) [36-38] to afford the nitronitrate product via rearrangement of the nitrosoperoxyl intermediate [6,26].

Under the experimental conditions reported in the present study, both the NO_2 and the O_2 coupling pathways are in principle viable routes to nitronitrate adducts. Arguments in favour of the former path include a greater kinetic constant for the coupling of NO_2 with alkyl radicals ($k=1.7 \times 10^{10}$ vs. $k=2 \times 10^8$ to 10^9 for O_2 at 25°C [21]) and the generation of thermodynamically stable termination products compared to peroxyl radicals. On the other hand, the reactions of NO_2 with olefins are known to give higher yields of nitronitrate derivatives in oxygenated media [11,12] and, when both NO_2 and O_2 are available, the latter appears to trap carbon-centred radicals more efficiently [21-24], which would tip the balance of available evidence in favour of the O_2-mediated pathway. This mechanistic analysis, however, assumes that in cyclohexane the potential contribution of heterolytic routes, e.g. the addition of N_2O_3 to double bonds, is negligible [17], which may not be the case, and studies are currently in progress to address this issue more specifically. In this frame, nitroalkene compounds would conceivably arise by loss of nitric acid from the vicinal nitronitrate adducts [17], from dinitro- or nitronitrites, possibly by concerted elimination via a highly favoured 6-membered transition state, and/or by decomposition of diazonium nitrates produced by addition of NO to putative nitronitroso intermediates [28,39]. Whatever the actual mechanism, formation of *E*- versus *Z*-nitroolefin products appears to be invariably favoured.

The dramatic change of mechanism in aqueous medium conceivably reflects the preponderance of NO_2-induced hydrogen abstraction from the doubly allylic methylene groups [23,24], apparently a minor route in cyclohexane, occurring probably on the surface of emulsion droplets and propagating through radical chain processes. The effects of solvation on the NO_2-initiated competition between H-atom abstraction and addition pathways had previously been addressed in comparison to gas phase reactions [22]. In solution, the addition equilibrium would conceivably be more shifted toward the reagents because of the expected stabilisation of the transition state by solvation, whereby allylic hydrogen abstraction mechanisms would prevail. Whether similar arguments can be invoked to explain the medium-induced effects on product distribution reported in the present study was investigated by a preliminary quantum mechanical approach aimed at exploring the effects of cyclohexane and water on the reactions of NO_2 with a model 1,4-diene, (*Z*,*Z*)-1,4-pentadiene (Figure 4).

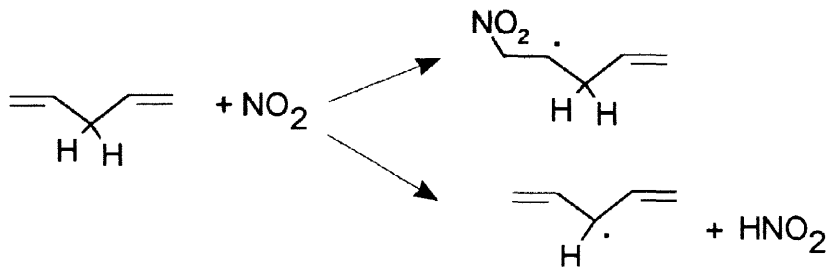


Figure 4. Addition and H-atom abstraction reactions of NO_2 with (*Z,Z*)-1,4-pentadiene

To this end, the Polarisable Continuum Model (PCM) implemented by one of us in the Gaussian 98 package [40,41] was employed. Calculated ΔG values (Table 3) indicated that the solvent facilitates the H-atom abstraction route compared to the gas phase reaction, and that H-atom abstraction is favoured over homolytic addition both in vacuo and in solution.

Table 3.
ΔG (kJ mol^{-1}) for the reaction of (*Z,Z*)-1,4-pentadiene with NO_2 calculated at the B3LYP/6-31G* (in vacuo) and the B3LYP/6-31G*/C-PCM (water and cyclohexane) levels.

	in vacuo	cyclohexane	water
ΔG addition	+6.11	-0.63	-21.80
ΔG abstraction	-3.09	-9.96	-26.48

In cyclohexane, moreover, addition would be almost completely reversible, as generally agreed, whereas in water the equilibrium would be more shifted toward the product, though H-atom abstraction would be still favoured because of the largest negative ΔG. The limitations inherent to 1,4-pentadiene as a model of long chain fatty acids, and the difficulties to reproduce theoretically the actual environment of a fatty acid in aqueous emulsion, where solvation may not be effective and there exists a hydrophobic region in which lipid chains act as a neat ordered liquid, should of course be considered. Nevertheless, this theoretical approach corroborates most of the previous observations on the reversibility of the addition reaction and the general irreversibility of H-atom abstraction. In fact, the apparent discrepancy with experimental data can be reconciled in terms of previous arguments suggesting that medium control over product distribution is exerted through an array of different factors acting in concert. Most likely, the kinetically faster addition reaction (for NO_2 k_{add}/k_{abs} is in the range of 10^3-10^6) [22] prevails in cyclohexane at high NO concentration, under conditions where β-nitroalkyl radicals can be efficiently captured by NO, O_2 or NO_2, and the resulting coupling products can be readily stabilised through subsequent irreversible rearrangement, scission/recombination or oxidation steps. By contrast, in aqueous medium, where NO solubility is one order of magnitude lower than in organic solvents [2,42], interception of β-nitroalkyl radicals in the addition route may be less efficient, whereas the H-abstraction path would be irreversibly driven by fast deprotonation

of the resulting HNO_2 at pH 7.4. Prevalent coupling of the resonance stabilised pentadienyl radical with O_2 rather than NO or NO_2, even with the relatively large concentrations of nitrogen oxides utilised in our experiments, is in line with previous observations [6,22,23,24] and accounts for the generation of a chain propagating peroxyl radical. Hydrolytic decays of N_2O_3 and N_2O_4 (if any), though slow, should be significant at pH 7.4, and would lower the concentrations of nitrogen oxides available for radical coupling.

In conclusion, the results described in this paper corroborate and integrate previous reports on the nitration of fatty acids with nitrogen oxides, addressing the behaviour of the core 1,4-diene moiety with high concentrations of NO and providing a complete spectral characterisation of a collection of nitrated derivatives somewhat different from those described in the previous studies.

3. Experimental

Arachidonic acid, methyl arachidonate and ethyl linoleate were purchased from Sigma. NO gas (electronic grade, 99.99%) was from Air Liquide and was purified from higher nitrogen oxides by passage through a solution of concentrated NaOH previously purged with Ar for 1 h and then through NaOH pellets. Griess reagent consisted of 1% sulphanilamide and 0.1% naphthylethylenediamine in 5% phosphoric acid [29]. Silica gel plates (0.25 and 0.5 mm, F254) were from Merck. Aqueous buffers were prepared in glass distilled, deionised water.

FT-IR spectra were determined on a Perkin Elmer 1760-X spectrophotometer. EI-MS spectra were determined with a Trio 2000 Fisons spectrometer at 70 eV. ^1H (^{13}C)-NMR spectra were recorded at 270 (67.9) or 400 (100) MHz on Bruker AC 270 or WM 400 spectrometers. 2D carbon-proton shift correlation experiments were performed at 100 MHz using a Bruker XHCORR microprogram. GC-MS was carried out on a Hewlett-Packard 5970 apparatus using a Supelco SPB-1 column (30m x 0.25 mm). Helium was the carrier and the flow rate was 0.8 ml/min. CAUTION! NO and related nitrogen oxides are highly toxic and all operations must be carried out under an efficient hood.

Reaction of arachidonic acid and methyl arachidonate with NO in cyclohexane

Purified NO gas was slowly bubbled for about 5 min into a vigorously stirred solution of arachidonic acid or methyl arachidonate (100 mg, $1.0x10^{-3}$ M) in anhydrous cyclohexane at 25 °C. Argon was then flushed through the solution for 10 min and the solvent evaporated to dryness at room temperature. In the case of methyl arachidonate, the yellowish residue was taken up in cyclohexane and chromatographed on silica plates (0.5 mm) using cyclohexane-ethyl acetate 7:3 v/v as the eluant. About 30 mg of unreacted substrate were recovered. The main Griess-positive band at R_f=0.65 was scraped off and eluted with ethyl acetate to give an oily residue (about 15 mg). FT-IR ($CHCl_3$) ν_{max} 1736, 1649, 1559, 1523, 1335 cm^{-1}. ^1H-NMR ($CDCl_3$) δ 0.88 (3H, m), 1.25 (6H, m), 1.71 (2H, m), 2.06 (3H, m), 2.32 (2H, m), 2.55 (0.5H, m), 2.74 (3H, m), 2.95 (1.5H, m), 3.35 (1H, m), 3.66 (3H, s, reference peak for integration),

5.20-5.80 (7.5H, m), 7.10 (0.5H, m). ^{13}C-NMR ($CDCl_3$): δ 14.7 (CH_3), 23.2-34.2 (groups of CH_2), 52.9 (CH_3O), 78-88 (groups of CH), 122-142 (groups of CH), 150-155 (groups of C), 175.5 (CO). EI-MS: m/z 346, 331, 317, 316, 306, 285, 276.

Reaction of methyl arachidonate with NO in phosphate buffer

Purified NO was slowly bubbled through a fine, milky suspension of methyl arachidonate ($1.0x10^{-3}$ M) in 0.1 M phosphate buffer, pH 7.4, obtained by adding dropwise a small volume (1-2%) of a concentrated methanol solution of the substrate to the buffer under vigorous stirring at 25°C. After 5 min, the reaction mixture was purged with argon, treated with $NaBH_4$ and extracted with cyclohexane. The organic phase was dried over anhydrous sodium sulphate and evaporated to dryness. The main UV absorbing products were isolated by preparative TLC (cyclohexane-ethyl acetate 7:3 v/v) and characterised by spectroscopic analysis and comparison of the chromatographic properties with those of the conjugated diene products formed by lipoxygenase or iron-induced oxidation of methyl arachidonate after treatment with $NaBH_4$. Selected spectroscopic data: UV (cyclohexane) λ_{max} 235 nm; ^{1}H-NMR (acetone d_6): δ (main resonances) 0.88 (3H, m), 1.29 (8H, m), 1.68 (2H, m), 2.10 (ca. 2H, m), 2.32 (2H, m), 2.78 (ca. 4H, m), 3.65 (3H, s), 4.32 (1H, m), 5.39 (ca. 5H, m), 5.65 (1H, dd, J=17,8 Hz, 6.02 (1H, dd, J=12,12 Hz), 6.62 (1H, dd, J=17,12 Hz).

Reactions of ethyl linoleate with NO

The reactions of ethyl linoleate with NO in cyclohexane [25] and in 0.1 M phosphate buffer, pH 7.4, were carried out as described above. The mixture obtained in aqueous buffer was treated with sodium borohydride and extracted with cyclohexane. TLC fractionation (cyclohexane-ethyl acetate 8:2 v/v) afforded a main UV-absorbing fraction (R_f=0.5) as a colourless oil (32% w/w) consisting of a mixture of ethyl hydroxyoctadecadienoates: UV (cyclohexane) λ_{max} 235 nm; ^{1}H-NMR ($CDCl_3$) δ 0.88 (3H, m), 1.25 (19H, m), 1.60 (2H, m), 2.10 (2H, m), 2.27 (2H, m), 4.10 (2H, q, J=7.5 Hz), 4.35 (1H, m), 5.51 (2H, m), 5.99 (1H, m), 6.26 (0.5H, dd, J=15,10 Hz), 6.52 (0.5H, dd, J=15,10 Hz).

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