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Oxidation of cholesterol and *O*-protected derivatives by the environmental pollutant $NO_2^{\bullet}$†

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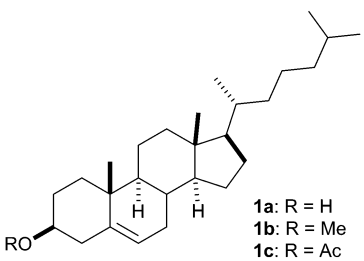
Exposure of *O*-protected and free cholesterol 1 to $NO_2^{\bullet}$ under exclusion of water leads to nitroimine nitrates 2 through a non-radical mechanism, which reveals the high susceptibility of the π system to oxidative damage. In the presence of moisture the reaction leads to 6-nitrocholesterols 3, which result from hydrolysis and oxidation of 2.

Nitrogen dioxide, $NO_2^{\bullet}$, is an important gaseous air pollutant, which is formed through combustion processes, for example car exhaust. This highly poisonous free radical is a promoter of oxidative stress,[1] and *in vivo* studies showed that $NO_2^{\bullet}$ exposure significantly reduces the levels of antioxidants (*e.g.*, glutathione, ascorbic acid, uric acid, *etc.*) in the airway surface fluids (ASF).[2] Such a weakened defence shield could provide a pathway for environmental oxidants to directly attack proteins and lipids present on cell surfaces or in the ASF. The resulting highly reactive oxidation products may subsequently damage the underlying epithelial cells and cause inflammation. However, despite the known adverse health effects, it is surprising that details of the processes that take place during the encounter of $NO_2^{\bullet}$ with ASF constituents are still not well understood at the molecular level.

We recently discovered an unexpected 'dual' reactivity of $NO_2^{\bullet}$ with oligopeptides. Thus, $NO_2^{\bullet}$ reacts as a radical oxidant [$E^0(NO_2^{\bullet}/NO_2^{\bullet}) = 1.03$ V][3] with peptides possessing readily oxidisable side chains, such as in tyrosine or tryptophan, which leads to formation of nitrotyrosines or pyrroloindolines, respectively.[4] Oligopeptides with non-oxidisable side chains, on the other hand, react with $NO_2^{\bullet}$ *via* its dimer N_2O_4 through non-radical *N*-nitrosation of peptide bonds. This triggers a rearrangement of the peptide backbone through formal excision of an amino acid moiety with concurrent joining of the remaining peptide ends to form a shortened peptide.[4]

Because of the only moderate water solubility of $NO_2^{\bullet}$ [$K_H(NO_2^{\bullet}) = 1.2 \times 10^{-2}$ M atm^{-1}, at 298 K],[6] the majority of inhaled $NO_2^{\bullet}$ is believed to reach the lower respiratory tract. In the lower airways cholesterol (**1a**) is the most abundant neutral lipid in the epithelial lining fluid,[7] which is directly exposed to oxidizing environmental pollutants.[8] Cholesterol is an integral component of the pulmonary surfactant (content about 5–10%)[9a] and essential for the integrity of cellular membranes by maintaining membrane fluidity and function.[9b] Interestingly, elevated cholesterol concentrations were found in the airway epithelial cells in mouse models of cystic fibrosis.[10]



Reaction of cholesterol with the non-radical air pollutant ozone has been shown to occur at the alkene moiety and leads to formation of bioactive oxysterols, which, if not eliminated, induce apoptosis and cytotoxicity.[7,11] A high reactivity towards $NO_2^{\bullet}$ has also been reported. *In vivo* exposure studies showed a significant reduction of the cholesterol content in the brains of guinea pigs.[12] Likewise, loss of cholesterol was also observed in cholesterol monomolecular films upon exposure to gaseous $NO_2^{\bullet}$.[13] However, only few studies were concerned with the identification of the reaction products and mechanism of their formation. Thus, when **1a** was exposed to $NO_2^{\bullet}$ in tetrachloromethane[14] or in hexane under varying conditions,[15] formation of cholesteryl nitrite (**1** with R = NO) was found as major pathway, in addition to by-products that result from reactions involving the C=C moiety.[14]

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† Electronic supplementary information (ESI) available: General experimental procedures, spectroscopic data for **1–3b,c**, crystallographic data for **2b**, Gaussian archive entries for the reaction **10** → **11**. CCDC 1436865. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c5cc09663d

Because of the two reactive sites in cholesterol, *i.e.*, the electron rich π system and the hydroxyl group, knowledge of the products formed in reactions with $NO_2^\bullet$ under simulated environmental conditions is essential for gaining mechanistic understanding and assessing the potential biological implications. We have therefore performed a product study of the reaction of $NO_2^\bullet$ with the 'isolated' π system using the *O*-protected cholesterols **1b** and **1c**, and with free cholesterol (**1a**). Different experimental conditions were employed: (i) the heterogeneous reaction of gaseous $NO_2^\bullet$ with solid **1b,c** in the absence and presence of moisture, and (ii) the homogeneous reaction of $NO_2^\bullet$ with **1a–c** in organic solvents, *e.g.*, dichloromethane or acetonitrile.

For the gas/solid exposure studies, solid **1b** or **1c** was placed in a reaction flask sealed with a silicon septum. Liquid $NO_2^\bullet$ (*ca.* 20 equiv.) was injected into an open vial standing upright in the reaction flask to avoid direct contact with the substrate. Evaporation created an atmosphere of $NO_2^\bullet$ gas, which reacted with **1b,c** at the gas/solid interface.‡ In order to simulate the conditions in airway surfaces, where the aqueous lining fluid containing cholesterol is exposed to gaseous $NO_2^\bullet$, we also performed experiments where the substrate was moistened with 40 equivalents of water prior to exposure to gaseous $NO_2^\bullet$.§ The reactions in organic solvents were carried out under exclusion of moisture by injecting a measured excess amount of liquid $NO_2^\bullet$ into the solution containing the substrate, as described previously.[4,5] For experimental details see ESI.† Product identification was performed by NMR, HRMS (ESI), IR and X-ray analysis (where possible).

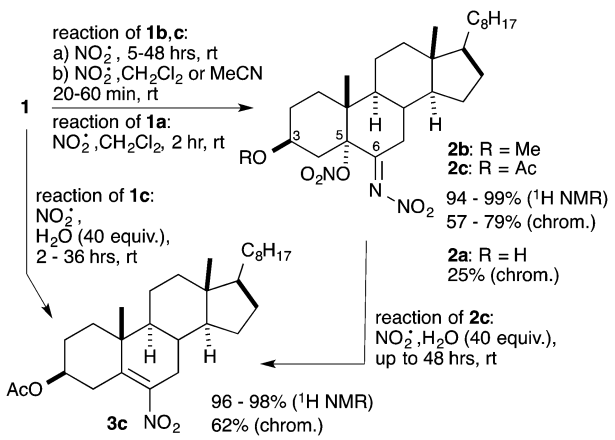
When solid *O*-protected cholesterols **1b** or **1c** were exposed to gaseous $NO_2^\bullet$ under exclusion of moisture a change of the substrate from a white solid to a green-blue oil occurred within few hours of contact. The reaction was very clean, and the ^{1}H NMR spectra of the respective raw reaction mixtures showed formation of the nitroimine nitrates **2b** and **2c** as only product (^{1}H NMR purity >94%), which did not undergo further reaction, even after an extended contact time of up to three days (Scheme 1). Due to some decomposition on the silica column, the isolated yield after purification was lower, although, once isolated, both **2b** and **2c** are stable compounds. HRMS analysis for **2b** revealed the protonated molecular ion $[M + H]^+$ at *m*/*z* 522.35370, which is consistent with the molecular formula $C_{28}H_{48}N_3O_6^+$ (calcd 522.35373) and confirms addition of three nitrogen and four oxygen atoms to the molecular framework. The ^{1}H NMR data revealed only loss of the vinyl proton at C-6, whereas the ^{13}C NMR spectrum showed the carbon atoms of the former alkene moiety at δ = 92.99 (C-5) and 176.01 ppm (C-6), respectively. Fortunately, **2b** crystallised upon standing. X-ray analysis (Fig. S14, ESI†) revealed a nitroimine group at C-6 and a nitrate ester at C-5, which sits *trans* to the angular methyl group and the oxygen substituent at C-3. Likewise, HRMS analysis for **2c** showed the protonated molecular ion $[M + H]^+$ at *m*/*z* 550.34955, which is consistent with the molecular formula $C_{29}H_{48}N_3O_7^+$ (calcd 550.34868). Although **2c** did not crystallise, the comparable spectroscopic data of **2b** and **2c** strongly support the presence of a similar nitroimine nitrate motif in **2c** (see ESI† for details).

The nitroimine nitrate ester was also exclusively formed, when exposure to gaseous $NO_2^\bullet$ occurred in the presence of air. This suggests an ionic mechanism, similar to our findings in the reaction of $NO_2^\bullet$ with peptides,[4,5] since intermediately formed radicals would be expected to react, at least in part, with oxygen in air, leading to different products.

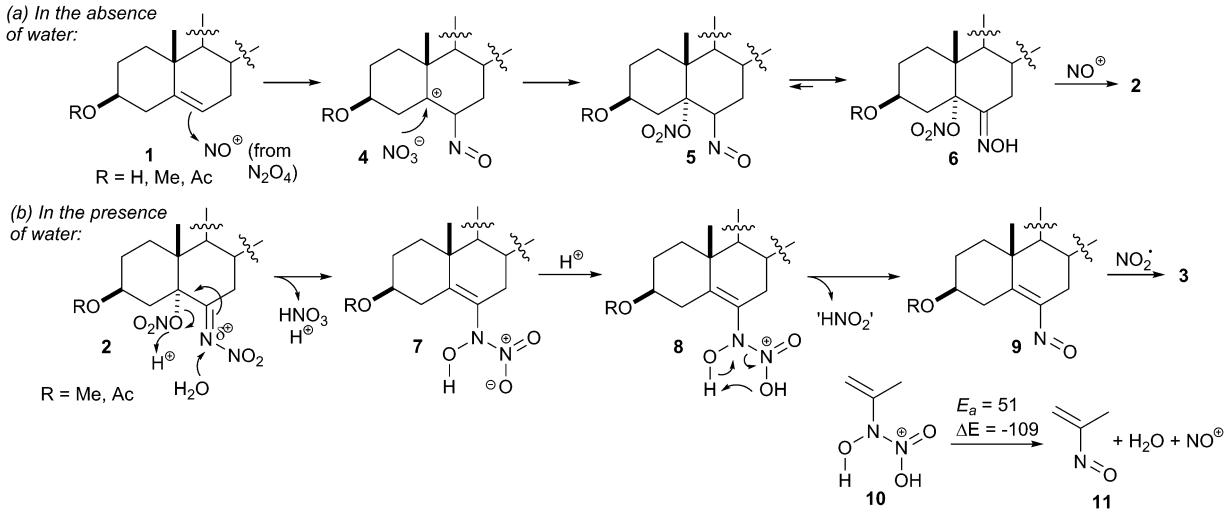
Interestingly, when the reaction was carried out as homogeneous reaction by dissolving **1b,c** and $NO_2^\bullet$ in dichloromethane or acetonitrile, the same outcome was obtained. Under these conditions, however, consumption of the starting material was complete after 20 minutes. Similar to the heterogeneous gas exposure studies, elongation of the reaction time did not lead to decomposition or further conversion of the reaction product **2**. This demonstrates that aprotic organic solvents provide a suitable and timesaving model environment for $NO_2^\bullet$ gas phase exposure studies under non-aqueous conditions. Finally, the role of $NO_2^\bullet$ as the sole species responsible for the transformation was undoubtedly confirmed by *in situ* ^{1}H NMR analysis of the reaction of **1b**, which revealed **2b** as the only product after 20 minutes of reaction time (see Fig. S12, ESI†).

The reaction of cholesterol (**1a**) with $NO_2^\bullet$ in dichloromethane gave a complex mixture of products, undoubtedly due to reactions involving the hydroxyl group. Fortunately, we were able to isolate the major product in 25% yield, which was unambiguously identified from the NMR, IR and HRMS data as the nitroimine nitrate **2a** (Scheme 1, see ESI† for details). This clearly shows that selective reaction of $NO_2^\bullet$ with the π system in **1a** occurs even in the presence of the hydroxyl group to a significant extent, which demonstrates the high susceptibility of the alkene moiety in cholesterol to oxidative damage. Gas/solid exposure studies with **1a** were also attempted, but the resulting product mixture was too complex to be analysed.

A mechanistic proposal for formation of **2** is outlined in Scheme 2a (picturing only the structural motif relevant to this reaction). We believe that the dimer N_2O_4 is the actual key-species in this process,[4,5] which is a non-radical nitrosating agent as revealed by its isomeric ionic form $[NO^+NO_3^-]$.[16] The reaction occurs by electrophilic addition of NO^+ to the C=C bond, which is immediately followed by recombination of the resulting cationic intermediate **4** with NO_3^- from the sterically



Scheme 1 Reaction of cholesterols **1a–c** with $NO_2^\bullet$.



Scheme 2 Proposed mechanism for formation of the nitroimine nitrate **2** and nitrocholesterol **3** in the reaction of cholesterols **1a–c** with $NO_2^\bullet$ in the absence (a) and presence of water (b). M06-2X/6-311++G** energies for the model reaction **10** → **11** in kJ mol^{-1}.

less hindered side to give the nitroso nitrate ester **5**. Tautomerisation of the nitroso group yields oxime **6**, which is subsequently converted to the nitroimine through reaction with NO^+.[17] The high efficiency of the trapping of **4** by NO_3^- was revealed through competition experiments performed with **1b** and $NO_2^\bullet$ in dichloromethane in the presence of excess acetic acid. ^{1}H NMR analysis of the product mixture indicated formation of the corresponding nitroimine acetate (not shown)[18] together with nitrate **2b** in a 1 : 10 ratio, which suggests that NO^+ and NO_3^- exist as contact ion pair with very limited solvent separation. It should be noted that the ionic character of N_2O_4 is a feature largely known for the condensed phase.[16] Our finding that **2** is formed in both the gas/solid reaction as well as in aprotic organic solvents, indicates that [$NO^+NO_3^-$] should also be the active species in our gas phase exposure studies. It could be speculated that dissociation into the ion pair occurs when gaseous $NO_2^\bullet/N_2O_4$ 'dissolves' in the solid substrate **1**, which could explain the colour change observed during the exposure (see above).

Interestingly, a completely different outcome was obtained when solid cholesterol acetate **1c**, which was moistened with water (40 equivalents), was exposed to gaseous $NO_2^\bullet$.¶ According to ^{1}H NMR analysis of the crude reaction mixture, practically quantitative conversion of **1c** to 6-nitro cholesterol **3c** occurred, which is stable under the reaction conditions for several days (up to 36 hours were explored).‖ The lack of a vinyl proton in the ^{1}H NMR spectrum of **3c** and the molecular ion of the dimer $[2M + Na]^+$ at *m*/*z* 969.68402 in the HRMS, which corresponds to the molecular formula $C_{58}H_{94}N_2O_8Na^+$ (calcd 969.69024), suggests substitution of the hydrogen atom at C-6 by NO_2.** Since the product did not crystallise, the structure of **3c** was assigned by comparing the NMR data with those of a reference sample, which was prepared by nitration of **1c** with sodium nitrite and nitric acid.[19] It should be noted that exposure of moistened cholesterylmethyl ether **1b** to gaseous $NO_2^\bullet$ resulted in ether cleavage and formation of various hydrophilic products, which could not be identified.††

At first sight, formation of **3c** might suggest a straightforward radical process, where addition of $NO_2^\bullet$ to the sterically less hindered site of the alkene moiety in **1c** is followed by hydrogen abstraction, for example by $NO_2^\bullet$,[20] to restore the π system (mechanism not shown). However, because of the unquestionable role of water in this transformation, we explored the mechanism in more detail. Thus, exposure of a 1 : 1 mixture of nitroimine nitrate **2c** and nitrocholesterol **3c** to gaseous $NO_2^\bullet$ in the presence of moisture resulted in complete conversion of **2c** into **3c** (see Fig. S13 in the ESI†). This provides strong support for **2c** being an intermediate in the $NO_2^\bullet$ mediated transformation **1c** → **3c**. No reaction took place when **2c** was treated with either water or with $NO_2^\bullet$ in the absence of moisture (see above). However, **3c** was also formed, when the reaction of $NO_2^\bullet$ was performed with **2c** that was dampened with methanol (not shown), illustrating the importance of a proton source for this transformation. On the other hand, although the system $NO_2^\bullet/N_2O_4$/water provides a considerably acidic environment, no reaction occurred when **2c** was treated with concentrated nitric acid in dichloromethane. This shows that an acidic environment alone is not responsible for the transformation **2** → **3**. Based on these observations a mechanism is proposed in Scheme 2b. Due to the electron-withdrawing nature of the nitro group, the imine nitrogen atom in **2** is positively polarised and could be attacked by water in a nucleophilic allylic substitution, where nitric acid acts as leaving group. Protonation of the nitro group in **7** enables a subsequent heterolytic N–N bond cleavage in **8** that yields nitroso compound **9** and nitrous acid. Density functional theory calculations for the simplified model system **10** → **11** at the M06-2X/6-311++G** level of theory showed that this process is not only associated with a modest activation barrier, E_a, of 51 kJ mol^{-1}, but is also considerably exothermic ($\Delta E = -109$ kJ mol^{-1}).‡‡ The resulting 6-nitroso cholesterol **9** is subsequently oxidised to the final product by $NO_2^\bullet$, which acts as one-electron oxidant in this step.[21]

In conclusion, the cholesterol alkene moiety is readily attacked by $NO_2^\bullet$ in both *O*-protected and free cholesterol. This reaction

involves N_2O_4 as key species, which acts as a non-radical nitrosating agent and leads to formation of nitroimine nitrate **2**. In the presence of moisture, **2** undergoes rapid hydrolysis and further $NO_2^{\bullet}$ mediated oxidation to give nitrocholesterol **3**. The latter would be the expected product in airway surface fluids, where it could serve as biomarker for $NO_2^{\bullet}$ pollution. In fact, nitrated lipids have been detected *in vivo*, where they play an important role in cell signalling.[22] Recent research revealed also beneficial effects in some nitrated lipids through their decomposition to release $NO^{\bullet}$, which causes vasorelaxation and has anti-inflammatory effects.[23]

The mechanism by which $NO_2^{\bullet}$ was found to react with the cholesterol π system in this work is considerably different from the radical addition/allylic hydrogen abstraction pathway proposed by Pryor *et al.* for the reaction of $NO_2^{\bullet}$ with cyclohexene, which was used as model system to study exposure of unsaturated fatty acids to air pollution.[20] The findings presented here, in particular the mechanistic studies, confirm our previous observations that exposure to $NO_2^{\bullet}$ leads to oxidative damage not only through radical pathways, but that ionic chemistry also contributes crucially to the reactivity of this important environmental pollutant and needs to be considered when assessing $NO_2^{\bullet}$ toxicity.[4,5] This conclusion is further supported by a recent suggestion that antioxidants mediate $NO_2^{\bullet}$ absorption in the ASF by catalysing its hydrolytic disproportionation to NO^+ and NO_3^-.[24] This could be the key to our understanding why $NO_2^{\bullet}$ has such pronounced biological effects *in vivo*, despite its low water solubility.

CAUTION: *N*-Nitroimines are known carcinogens. Proper precautions against inhalation of the vapours and contact with skin should always be maintained.

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Notes and references

‡ The reaction was quenched by removing unreacted $NO_2^{\bullet}$ through a stream of nitrogen, followed by dissolving the reaction mixture in dichloromethane and concentration *in vacuo*. Aqueous work-up, where the reaction was first quenched with saturated sodium bicarbonate solution, followed by extraction with dichloromethane, gave the same outcome (see ESI†).

§ Because of the low solubility of **1b/c** in water, it was not possible to study the reaction of $NO_2^{\bullet}$ in the aqueous phase.

¶ Due to the complexity of the reaction involving **1a** under anhydrous conditions, the reactions in presence of moisture were only performed with the *O*-protected cholesterols.

‖ It was found that reaction of **1c** with $NO_2^{\bullet}$ to **3c** requires only trace amounts of moisture.

** The ion of the sodium adduct of the dimer was significantly more abundant in the mass spectrum of **3c** than that of the monomer. The latter could be produced through collision induced dissociation as $[M + Na]^+$ at *m*/*z* 496.33850 in the HRMS (ESI), which is in accordance with the molecular formula $C_{29}H_{48}NO_4Na^+$ (calcd 496.34756).

†† Ether cleavage is likely a result of the acidic environment of this reaction system.

‡‡ The reaction energy is calculated for the product association complex [**11**–H_2O–NO^+].

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