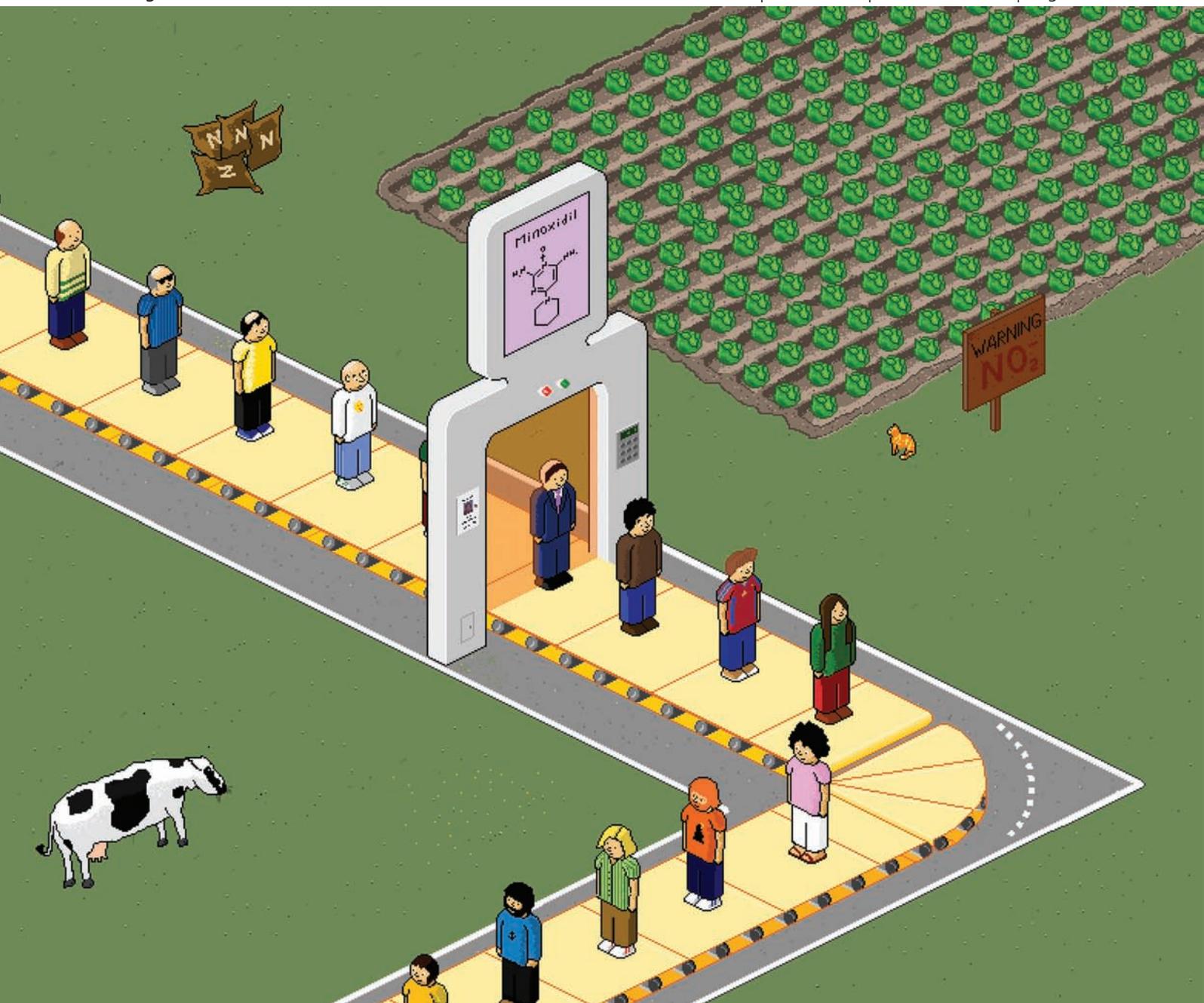


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Aromatic C-nitrosation of a bioactive molecule.
Nitrosation of minoxidil

Aromatic C-nitrosation of a bioactive molecule. Nitrosation of minoxidil†

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Minoxidil (2,4-diamino-6-(piperidin-1'-yl)pyrimidine *N*(3)-oxide; CASRN 38304-91-5) is a bioactive molecule with several nitrosatable groups widely used as an antihypertensive and antialopecia agent. Here the nitrosation of minoxidil was investigated. The conclusions drawn are as follows: (i) In the pH = 2.3–5.0 range, the minoxidil molecule undergoes aromatic C-nitrosation by nitrite. The dominant reaction was C-5 nitrosation through a mechanism that appears to consist of an electrophilic attack on the nitrosatable substrate by $\text{H}_2\text{NO}_2^+/\text{NO}^+$, followed by a slow proton transfer; (ii) the reactivity of minoxidil as a C-nitrosatable substrate proved to be 7-fold greater than that of phenol, this being attributed to the preferred *para*- and *ortho*-orientations of the two $-\text{NH}_2$ groups at positions 2 and 4 of the minoxidil molecule, which activate electrophilic substitution in the C-5 position through their mesomeric effect. The *N*-nitrosominoxidil resulting from the nitrosation could be potentially harmful to the minoxidil users.

1 Introduction

The report by Magee and Barnes¹ that dimethylnitrosamine induces liver cancer when fed to rats has prompted the study of the chemistry and biology of nitroso compounds. Biologists are mainly interested in the use of these compounds as models for producing a broad range of cancers^{2–9} whereas chemists are more interested first in the mechanisms of formation of nitroso compounds^{10–15} and then in blocking or inhibiting such mechanisms of these species.^{16–18}

N-Nitrosation chemistry has been a fruitful area for the mechanistic organic and physical organic chemist. Many reactions are now well understood, and in several cases the nature of the effective nitrosating species has been established, often by kinetic procedures.¹⁰

By contrast, aromatic C-nitrosation is much less known and has been investigated in relatively few cases. The reason for this is that substantial activation by electron-releasing groups is necessary to produce nitrosation at a suitable reaction rate.

The molecular structure of the minoxidil (**mnx**) molecule (2,4-diamino-6-(piperidin-1'-yl) pyrimidine *N*(3)-oxide; CASRN 38304-91-5; Fig. 1) means that this substance is very likely to undergo C-nitrosation at its C-5 position.

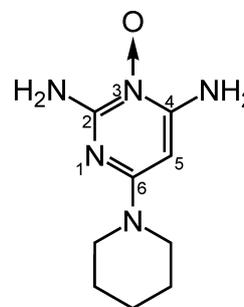


Fig. 1 Structure of minoxidil and IUPAC numeration of the atoms of pyrimidine ring.

In addition to its particular structure, minoxidil is a drug which has proved to be efficacious in patients with severe and drug-resistant forms of hypertension,¹⁹ and it is also widely used topically against androgenic or androgenetic alopecia.²⁰

Since: i) The bioactive minoxidil molecule could give nitrosation products potentially harmful to the patients treated with it, and ii) to our knowledge, the nitrosation of minoxidil has not yet been investigated, here we were prompted to investigate this issue.

2 Results and discussion

2.1 Kinetics and mechanism

The nitrosation of minoxidil was carried out with sodium nitrite (**nit**) in perchloric aqueous solutions at pH = 2.3–7.5. A significant nitrosation reaction was only observed in the pH = 2.3–5.0 range. Using the initial-rate method,²¹ a first-order reaction with respect to the minoxidil concentration was observed (eqn (1) and Fig. 2).

$$\text{initial rate} = a[\text{mnx}] \quad (1)$$

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† Electronic supplementary information (ESI) available: ¹H NMR, ¹³C NMR spectra of minoxidil. ¹H NMR, ¹³C NMR, 2D ROESY NMR, 2D COSY NMR spectra and crystallographic information file (CIF) of synthesized product. Mass spectrum from the nitrosation reaction. CCDC reference number 819626. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c1ob05686g

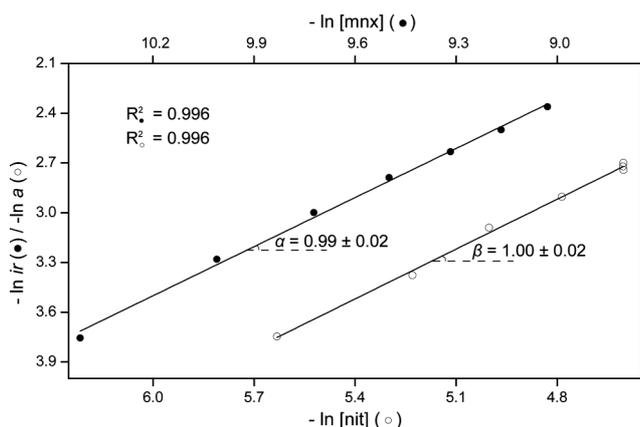


Fig. 2 Partial orders of reaction with respect to the concentration of minoxidil (α , [nit]₀ = 0.02 M) and to the nitrite (β). pH = 4.00, $T = 20^\circ\text{C}$, $I = 0.2\text{ M}$.

Experiments carried out with different excesses of sodium nitrite showed a first-order reaction (eqn (2)) with respect to its concentration (Fig. 2):

$$a = b[\text{nit}] \quad (2)$$

where a and b are experimental first-order rate constants.

To check that the order with respect to concentration coincided with the order with respect to time,²² experiments with equimolar concentrations (c) of nitrite and minoxidil were performed. Fig. 3 shows the good fit of the results to the integrated form of eqn (3), and reveals a global order two for the nitrosation reaction. This is consistent with no change in the reaction mechanism along the time of monitoring.

$$\text{rate} = -\frac{dc}{dt} = k_{\text{obs}}[c]^2 = k_{\text{obs}}[\text{nit}][\text{mnx}] \quad (3)$$

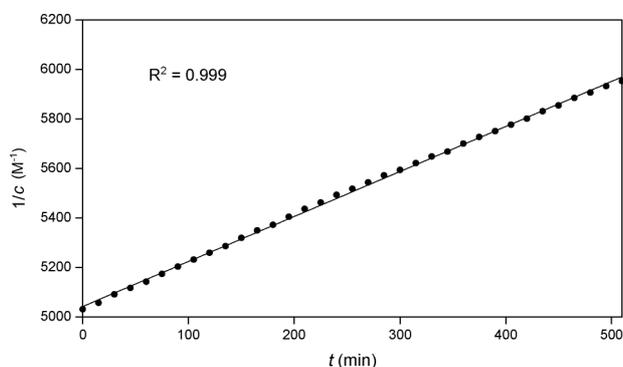


Fig. 3 Integrated form of the second-order rate equation (eqn (3)) for the reaction between minoxidil and nitrite. [nit]₀ = [mnx]₀ = $c_0 = 2 \times 10^{-4}\text{ M}$, pH = 4.00, $T = 20^\circ\text{C}$, $I = 0.2\text{ M}$.

On the basis of these results, and taking into account the absence of order two with respect to the nitrite concentration (*viz.*, the exclusion of dinitrogen trioxide as an effective nitrosating agent¹¹) and the lack of influence of ionic strength on k_{obs} (Fig. 4), a mechanism (Scheme 1) of aromatic electrophilic substitution by $\text{H}_2\text{NO}_2^+/\text{NO}^+$ (kinetically indistinguishable) whose rate-determining step is the deprotonation of the Wheland intermediate (*viz.*, nitrosoarenium, Scheme 1, II)^{18,23,24} can be

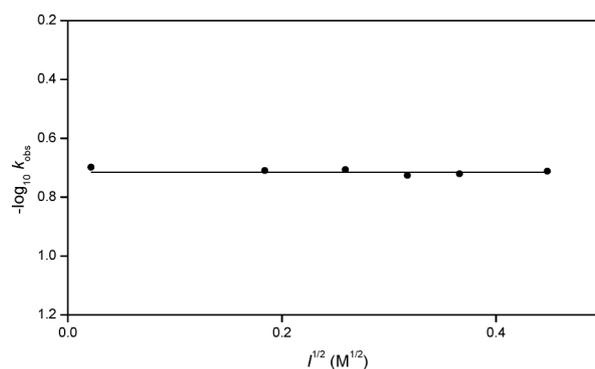
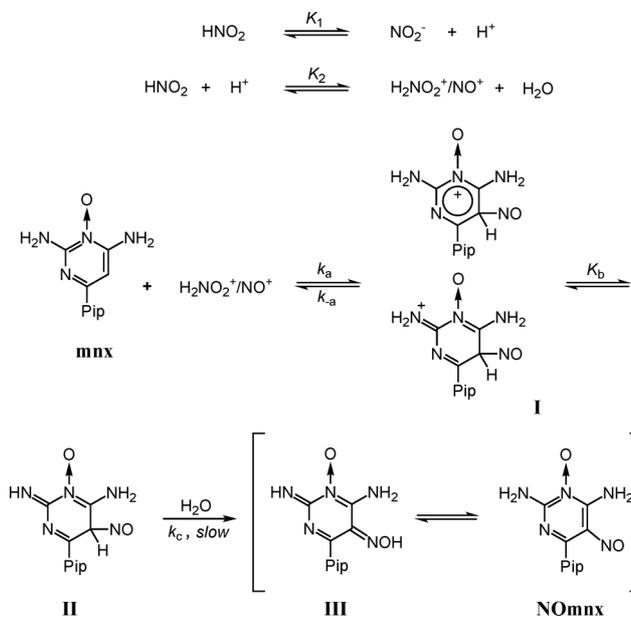


Fig. 4 Influence of ionic strength on the rate constant of nitrosation k_{obs} . [nit]₀ = [mnx]₀ = $2 \times 10^{-4}\text{ M}$, pH = 4.00, $T = 20^\circ\text{C}$.



Scheme 1 Reaction mechanism for the nitrosation of minoxidil.

proposed. Direct observation of the Wheland intermediate in electrophilic aromatic substitution (formation of nitrosoarenium cations) has been performed previously²⁵ using transient absorption spectroscopy.

From this mechanism, the rate eqn (4) is readily achieved.

$$\text{rate} = \frac{K_2 k_a [\text{nit}][\text{mnx}][\text{H}^+]^2}{([\text{H}^+] + K_1)(1 + \frac{k_{-a}}{K_b k_c} [\text{H}^+])} \quad (4)$$

Comparison of the experimental eqn (3) and theoretical eqn (4) rate equations implies:

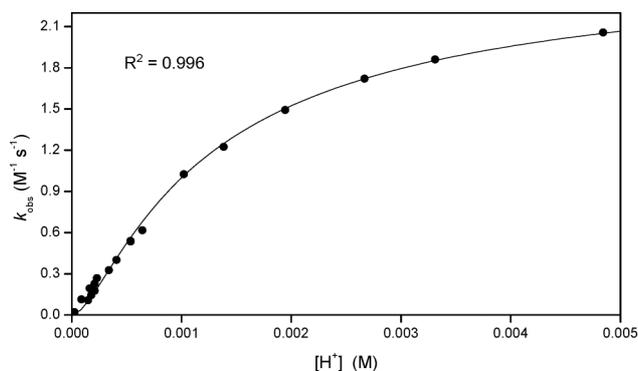
$$k_{\text{obs}} = \frac{K_2 k_a [\text{H}^+]^2}{([\text{H}^+] + K_1)(1 + \frac{k_{-a}}{K_b k_c} [\text{H}^+])} \quad (5)$$

Fig. 5 shows the excellent fit of the values of the experimental rate constant (k_{obs}) to eqn (5). From the least-squares fit, where K_1 was fixed ($K_1 = 6.652 \times 10^{-4}\text{ M}^{26}$) and $[\text{H}^+]$ was the variable, the $K_2 k_a$ and $k_{-a}/K_b k_c$ values were calculated (Table 1). Given that $K_2 = 3 \times 10^{-7}\text{ M}^{-1}$,²⁷ the value $k_a = (1.5 \pm 0.1) \times 10^{10}\text{ M}^{-1}\text{s}^{-1}$ is easily

Table 1 Kinetic isotope effect, $k_c^{\text{H}_2\text{O}}/k_c^{\text{D}_2\text{O}}$, for the nitrosation of minoxidil compared with those for other C-nitrosatable compounds

Substrate	$k_a \times 10^{-9} \text{ (M}^{-1} \text{ s}^{-1})^a$	$\frac{k_{-a}}{K_b k_c} \times 10^{-4} \text{ (M}^{-1})^a$	$K_2^{\text{H}_2\text{O}}/K_2^{\text{D}_2\text{O}}$
Minoxidil ^b	15 ± 1	0.17 ± 0.01	6.8 ± 0.1
Phenol ^c	2.2 ± 0.02	6.3 ± 0.5	3.5 ± 0.3
<i>o</i> -Cresol ^c	2.4 ± 0.3	1.0 ± 0.2	3.2 ± 0.3
2,3-Dimethylphenol ^c	3.6 ± 0.2	1.1 ± 0.2	3.0 ± 0.3
<i>o</i> -Chlorophenol ^c	0.33 ± 0.03	2.0 ± 0.2	2.2 ± 0.2

^a Values are given with their standard deviations. ^b This work. ^c Values taken from González-Mancebo *et al.*¹⁸

**Fig. 5** Fitting of the experimental rate constant for the nitrosation of minoxidil to the theoretical equation (eqn (5)). Dots are experimental values. Solid line fits eqn (5). $[\text{nit}]_0 = [\text{mnx}]_0 = 2 \times 10^{-4} \text{ M}$, $\text{pH} = 4.00$, $T = 20 \text{ }^\circ\text{C}$, $I = 0.2 \text{ M}$.

calculated. The values for other C-nitrosatable compounds¹⁸ are also shown in Table 1.

On the basis of the π electronic structures of the nitrosatable substrates these results are consistent with an aromatic electrophilic substitution mechanism (Scheme 1). The two $-\text{NH}_2$ groups of the minoxidil molecule activate electrophilic aromatic substitution in their respective *ortho* and *para* positions through their mesomeric effect. Since in both cases the activated position is the same (position 5, Fig. 1), the electrophilic aromatic substitution in that position should be reinforced.

As is well known, a primary kinetic isotope effect (KIE) can be detected by kinetic measurement only if the bond to the isotopically labelled atom is broken during or before the rate-determining step.^{28–31} This means that the C–H proton transfer involved in the slow kinetic step (k_c in Scheme 1) should show a primary isotope effect: $k_c^{\text{H}_2\text{O}}/k_c^{\text{D}_2\text{O}} > 1$. Since kinetic deuterium isotope effects have been observed previously during the nitrosation of several aromatic and heteroaromatic substrates,^{18,32,33} eqn (5) was used to check the existence of a primary KIE. Taking into account that under adequate pH conditions ($k_a/K_b k_c [\text{H}^+] \gg 1$ and $[\text{H}^+] \gg K_1$, eqn (5) leads to the expression:

$$\frac{k_{\text{obs}}^{\text{H}_2\text{O}}}{k_{\text{obs}}^{\text{D}_2\text{O}}} = \frac{K_2^{\text{H}_2\text{O}} k_c^{\text{H}_2\text{O}}}{K_2^{\text{D}_2\text{O}} k_c^{\text{D}_2\text{O}}} \quad (6)$$

Since the average value of $K_2^{\text{H}_2\text{O}}/K_2^{\text{D}_2\text{O}}$ is known (eqn (6)),³⁴ knowledge of $k_c^{\text{H}_2\text{O}}/k_c^{\text{D}_2\text{O}}$ is immediate. The KIE $k_c^{\text{H}_2\text{O}}/k_c^{\text{D}_2\text{O}} \approx 7$ (Table 1), which indicates that proton loss from the Wheland intermediate is the slow rate-determining step, is characteristic of aromatic electrophilic substitution reactions.²⁹

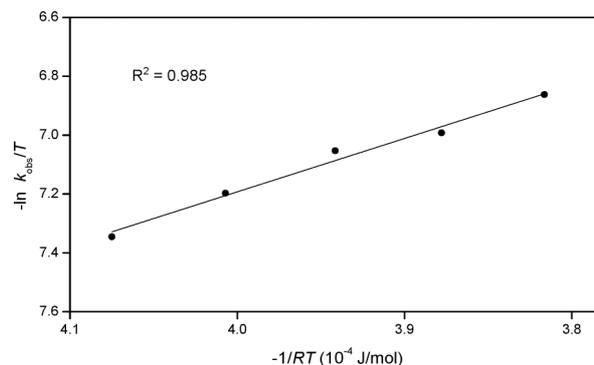
Table 2 Variation in the rate constant k_{obs} with temperature

$T/^\circ\text{C}$	$k_{\text{obs}} \times 10^2 \text{ (M}^{-1} \text{ s}^{-1})^a$
20	19.05 ± 0.03
25	22.46 ± 0.04
30	26.38 ± 0.05
35	28.50 ± 0.05
40	32.98 ± 0.06

^a Values are given with their standard deviations.

Furthermore, the existence of an isokinetic relationship can be used to support the argument that the reactions of a series of reagents share a common mechanism.^{35–37} To test this possibility, the activation parameters ΔH^\ddagger and ΔS^\ddagger were calculated with the values of k_{obs} measured at different temperatures (Table 2 and Fig. 6) using the Eyring equation,³⁸

$$\ln \frac{k_{\text{obs}}}{T} = \ln \frac{k}{h} + \frac{\Delta S^\ddagger}{R} - \frac{\Delta H^\ddagger}{RT} \quad (7)$$

**Fig. 6** Eyring plot for the determination of the activation parameters (ΔH^\ddagger , ΔS^\ddagger) for the minoxidil nitrosation reaction. $[\text{nit}]_0 = [\text{mnx}]_0 = 2 \times 10^{-4} \text{ M}$, $\text{pH} = 4.00$, $T = 20 \text{ }^\circ\text{C}$, $I = 0.2 \text{ M}$.

With the ΔH^\ddagger and ΔS^\ddagger values obtained here (eqn (7)) for the nitrosation of minoxidil ($\Delta H^\ddagger = 18 \pm 1 \text{ kJ mol}^{-1}$; $\Delta S^\ddagger = -163 \pm 20 \text{ J mol}^{-1} \text{ K}^{-1}$) and those previously determined for a series of C-nitrosation reactions occurring through electrophilic attack on the nitrosatable substrates by $\text{H}_2\text{NO}_2^+/\text{NO}^+$,¹⁸ the plot $\Delta H^\ddagger/\Delta S^\ddagger$ was drawn (Fig. 7). The results are consistent with a common mechanism.

2.2 Product Identification and Characterization

Nitrosominoxidil (**NOmnx**) was synthesized following the procedure reported in the Experimental Section. X-ray diffraction analysis showed that minoxidil is nitrosated at position C-5 of the pyrimidine ring (Fig. 8). Comparison of the NMR spectra (in the supporting information) before and after nitrosation confirms that position as center of nitrosation.

In order to check that the product of the minoxidil nitrosation was the same as that of previously synthesized nitrosominoxidil, the UV-spectra and the values of their respective molar absorption coefficients were determined (Table 3 and Fig. 9). In addition, the two samples were compared using an Ultra Fast Liquid Chromatography technique (ULFC). Using a linear gradient

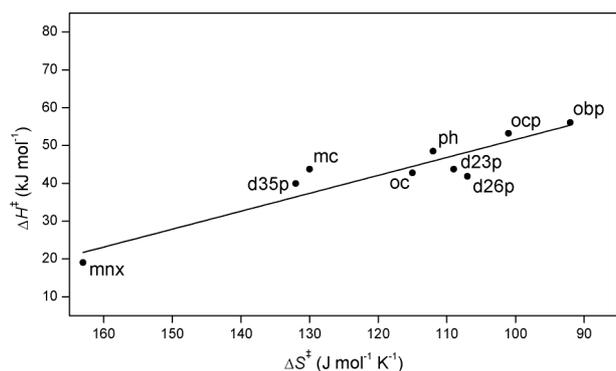


Fig. 7 $\Delta H^\ddagger/\Delta S^\ddagger$ isokinetic relationship for the C-nitrosation reactions of minoxidil (mnx) and other nitrosatable substrates: phenol (ph), *m*-cresol (mc), *o*-cresol (oc), 2,3-dimethylphenol (d23p), 2,6-dimethylphenol (d26p), 3,5-dimethylphenol (d35p), *o*-chlorophenol (ocp) and *o*-bromophenol (obp).¹⁸

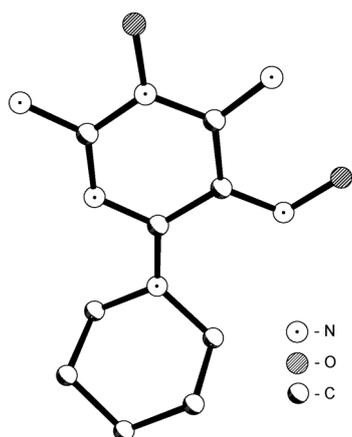


Fig. 8 X-Ray structure section showing the site where minoxidil is nitrosated.

Table 3 Molar absorption coefficients for minoxidil, nitrosominoxidil synthesized (*syn*) and nitrosominoxidil formed along the nitrosation reaction (*kin*)

Compound	λ (nm)	ϵ ($M^{-1} \text{ cm}^{-1}$) ^a	$T/^\circ\text{C}$	pH
NOmnx (<i>syn</i>)	321	$14,100 \pm 100$	20	4.08
NOmnx (<i>kin</i>)	321	$14,070 \pm 80$	20	4.12
NOmnx (<i>syn</i>)	400	$4,210 \pm 40$	20	4.08
NOmnx (<i>kin</i>)	400	$4,180 \pm 30$	20	4.12
mnx	282	$22,400 \pm 100$	20	3.90

^a Values are given with their standard deviations.

program starting at 5 min, from 10% to 100% of acetonitrile over 32 min resolved the major peak from both samples. The UV-visible spectrum from this peak corresponded to NOmnx. Also, a positive-mode electrospray ionization mass spectrum of a sample from the nitrosation reaction was obtained, affording a mass/charge ratio = 239.1, which is coherent with the structure of the resulting nitrosocompound.

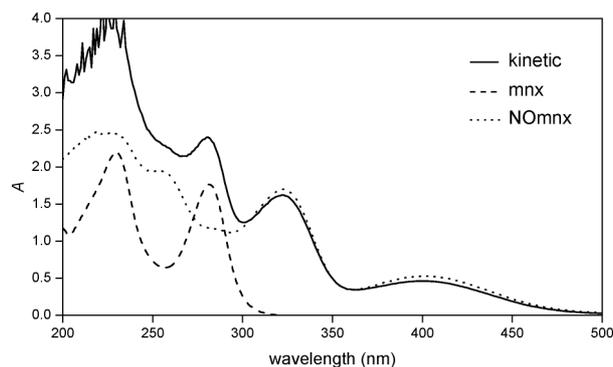


Fig. 9 UV-spectra of minoxidil (mnx), synthesized nitrosominoxidil (NOmnx), and nitrosominoxidil formed along the nitrosation reaction of minoxidil ($[\text{nit}]_0 = [\text{mnx}]_0 = 2 \times 10^{-4} \text{ M}$). All spectra are depicted at pH = 4 and $T = 20^\circ\text{C}$.

3 Conclusions

From the present work, the following conclusions were drawn:

(i) In the pH = 2.3–5.0 range, the minoxidil molecule undergoes aromatic nitrosation by nitrite. The dominant reaction is C-5 nitrosation through a mechanism that appears to consist of an electrophilic attack on the nitrosatable substrate by $\text{H}_2\text{NO}_2^+/\text{NO}^+$, followed by a slow proton transfer.

(ii) The reactivity of minoxidil as a C-nitrosatable substrate is 7-fold greater than that of phenol, this being attributed to the preferred *para*- and *ortho*-orientations of the two $-\text{NH}_2$ groups at positions 2 and 4 of the minoxidil molecule, which activate electrophilic substitution at position C-5 through their mesomeric effect.

(iii) A $\Delta H^\ddagger/\Delta S^\ddagger$ isokinetic relationship was found for the C-nitrosation reaction of minoxidil and other C-nitrosation reactions studied previously, occurring through electrophilic attack on the nitrosatable substrate by $\text{H}_2\text{NO}_2^+/\text{NO}^+$, followed by a slow proton transfer. This supports the idea of a common mechanism.

4 Experimental

4.1 General

Nitrosation reactions were followed by UV-visible spectrography, measuring the absorbance (A ; Fig. 9) of the reaction product ($\lambda = 321 \text{ nm}$) with a spectrophotometer equipped with a thermoelectric six-cell holder temperature-control system ($\pm 0.1^\circ\text{C}$). D-mnx was obtained by deuteration of H-mnx with D_2O . Proton and carbon NMR spectra were recorded in CD_3OD with a 400 MHz spectrometer. UFLC separations were performed with a 100 mL injection volume on a reversed-phase C18 column 5 mm, $250 \times 10 \text{ mm}$, attached to a guard C18 column, using a gradient-controlled UFLC system equipped with a diode array detector and channel UV light detection at 320 nm. The column oven was set at 40°C and the temperature of the diode array cell was 40°C . H_2O and acetonitrile were used as the mobile phases and the flow rate was 1 mL/min.

4.2 Synthesis of nitrosominoxidil

Nitrosominoxidil was synthesized as follows: 4.9 g of minoxidil (23 mmol) were dissolved in 17 mL of THF and then 3 mL of

fluoboric acid (HBF₄, 48%) was added. The mixture was cooled to below 0 °C and an ice-cold sodium nitrite solution (1.6 g, 24 mmol) in water (7 mL) was added dropwise during continuous stirring. After several hours standing, the nitrosominoxidil precipitated as greenish crystals. After vacuum filtration, the crystals collected were repeatedly washed with acetonitrile and ether and then desiccated. A portion of the product was recrystallized in methanol to perform the determination tests.

Yield: 92%; m. p.: 180 °C; ¹H NMR (400 MHz, CD₃OD) δ (ppm) 1.73 (m, 4H), 1.78 (t, *J* = 2.4 Hz, 2H), 4.13 (t, *J* = 3.6 Hz, 4H); ¹³C NMR (400 MHz, CD₃OD) δ (ppm) 25.6, 27.8, 139.8, 143.8, 154.4, 157.9; MS (EI) *m/z* 239.1

4.3 X-Ray Crystallographic Analysis of Nitrosominoxidil

A suitable single crystal of the compound was mounted on glass fibre for data collection on a diffractometer. Data were collected at 273 K using Cu-K_α radiation ($\lambda = 1.54178 \text{ \AA}$) and the ω scan technique, and were corrected for Lorentz and polarization effects. Structure solution, refinement, and data output were carried out with the SHELXTL™ program package. The structures were solved by direct methods combined with difference Fourier synthesis and refined by full-matrix least-squares procedures, with anisotropic thermal parameters in the last cycles of refinement for all non-hydrogen atoms. Hydrogen atoms were placed at calculated positions. Hydrogen atoms from the solvent molecules were located directly on a difference Fourier map.

a) Crystal data for nitrosominoxidil: 4(C₉H₁₄N₆O₂) × 3 H₂O, *M* = 1007.10, triclinic, space group *P*¹1 (*n*^o 2), *a* = 10.2884(3) Å, *b* = 13.6490(3) Å, *c* = 18.6062(5) Å, α = 110.437(2)°, β = 95.594(2)°, γ = 91.674(2)°, *V* = 2431.07(11) Å³, *Z* = 2, *D*_c = 1.376 Mg m⁻³, *m* = (Cu-K_α) = 0.886 mm⁻¹, *F*(000) = 1068. 9880 reflections were collected at 2.55 ≤ 2θ ≤ 50.17 and merged to give 4834 unique reflections (*R*_{int} = 0.0211), of which 4285 with *I* > 2σ(*I*) were considered to have been. The final values were *R* = 0.0414, *wR* = 0.1096, GOF = 1.037, max/min residual electron density 0.288 and -0.205 e. Å⁻³.

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