A mechanistic study on the oxidation of hydrazides: application to the tuberculosis drug isoniazid† ‡

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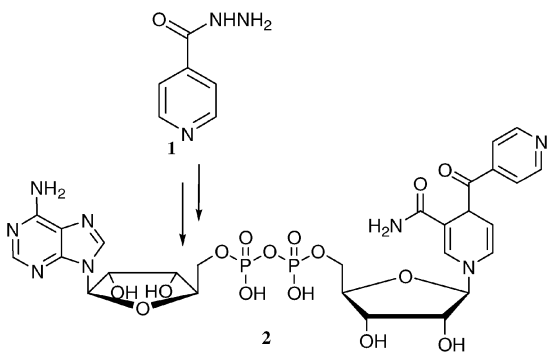
Herein we report radical trapping experiments that support the formation of an acyl radical as the active species from the oxidation of isoniazid; these data provide insight into the mechanism of hydrazide oxidation.

The Tuberculosis (TB) drug isoniazid (**1**, Scheme 1) has been used as a frontline treatment against the bacteria *Mycobacterium tuberculosis* for almost half a century.[1] It is only in the latter part of this period that studies have started to elucidate the complex mode of action in which isoniazid is active against the bacterium. A major driving force for this was the desire to develop an understanding that could be used to combat multi-drug resistance.[2] It was well established that isoniazid itself is not the true drug but that it is activated by oxidation to the active species. Electron spin resonance (ESR) studies have indentified radicals as intermediates in the activation[3] but is was not until Sacchettini reported the crystal structure of isonicotinic acyl-NADH (**2**, Scheme 1) in the active site of the enzyme InhA (part of the FASII pathway involved in building the bacterial cell wall) that it was confirmed that a reactive intermediate from isoniazid and NAD^+ combined to give the true inhibitor of the bacterium (Scheme 1).[4] It has been postulated that an acyl radical is the key intermediate[5] in the oxidation of isoniazid but to date this has not been verified.

When we first began this work little was known about the mechanism for the oxidation of hydrazides although it was well established that an electrophilic species is generated that has been exploited for acyl nucleophilic substitution reactions.[6,7] Only recently, when Braslau oxidized aryl and aliphatic hydrazides with PbO_2 in the presence of the radical trap 2,2,6,6,-tetramethylpiperidine-1-oxyl (TEMPO, **3**, Fig. 1), was it shown that the oxidation of hydrazides with transition metal oxidants involves the formation of an acyl radical intermediate.[8] It also seems generally accepted that an acyl radical could be further oxidized to the acyl cation.[5b,6,9] We now report preliminary results which suggest that acyl radicals are formed when hydrazides are oxidised by most oxidants and that the corresponding acyl cations do not play a major role in this pathway.

When benzhydrazide and isoniazid were reacted with various oxidants in the presence of TEMPO (**3**) under aqueous conditions *O*-benzoylhydroxylamine **4** and *O*-isonicotinoyl hydroxylamine **5** were formed, respectively (Fig. 1). These products are the consequence of acyl radical generation (**7a, 7b** Scheme 2) and trapping, as supported by Braslau.[8] The yields appeared to vary depending on the strength of the oxidant (Table 1, 8–84%). Significantly, these results suggest that acyl radicals are formed when either typical single electron oxidants or potential two electron oxidants were used.[10] This supports the previous hypothesis for isoniazid activation. The product was confirmed by NMR, IR and mass spectrometry. The other product formed in these reactions was the corresponding carboxylic acid (benzoic and isonicotinic) which were previously believed to result from acyl cation formation (**8a, 8b** Scheme 2) followed by reaction with water. The yields of **5** are much lower than for the corresponding



Scheme 1 Isoniazid (**1**) combines with NAD^+ under oxidative conditions to form the true inhibitor of InhA **2**.

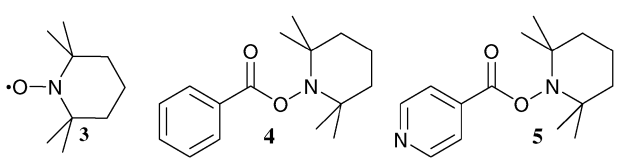


Fig. 1 The stable radical 2,2,6,6,-tetramethylpiperidine-1-oxyl (TEMPO) (**3**), and the resulting trapped products *O*-benzoylhydroxylamine **4** and *O*-isonicotinoylhydroxylamine **5**.

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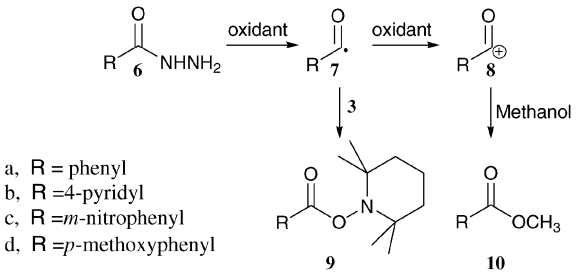


Scheme 2 Competitive trapping of radical with TEMPO (**3**), resulting in a TEMPO adduct, and cation with methanol, resulting in a methyl ester.

Table 1 Yields of trapped benzoyl and isonicotinoyl radical formed by various oxidants

Oxidant	Yield (%) **4**	Yield (%) **5**
$Mn(OAc)_3$	20	9
MnO_2	81	8
K_2MnO_4	67	10
$KMnO_4$	43	15
$[Mn_2(\mu\text{-}O)_3L_2](PF_6)_2$–$H_2O_2$	35	14
$[Mn_2(\mu\text{-}O)_3L_2](PF_6)_2$–$H_5IO_6$	21	58[a]
$K_3[Fe(CN)_6]$	84	20

[a] Nitrogen continuously bubbled through reaction mixture.

benzoyl derivative **4** (Table 1) indicating that the acyl radical from isoniazid (**7a**) is much less stable than that derived from benzhydrazide (**7b**) and was most likely oxidised further to the cation (**8b**, Scheme 2).

For our further investigations we used the manganese containing oxidant, $[Mn^{IV}$–$Mn^{IV}(\mu\text{-}O)_3L_2](PF_6)_2$ (L = 1,4,7-trimethyl-1,4,7-triazacyclononane) which we hypothesized as a mimic for oxidation by the KatG enzyme.[11] Under a nitrogen atmosphere only a modest yield of **5** was observed (9–20%), however, when the reaction solution was purged with nitrogen throughout the reaction, the yield was dramatically increased to 58% (See Table 1). One reason for this increased yield may be that as the manganese catalyst is a mimic for the oxygen evolving centre of photosystem II[12] it could generate a low concentration of oxygen in the presence of periodic acid.[11,13] Purging the reaction mixture with nitrogen gas eliminated this competing radical reaction such that **5** is now the major product. These experiments demonstrate that, under the conditions described above, the major oxidation pathway most likely involves formation of the acyl radical **7** (Scheme 2).

A competitive trapping scheme (Scheme 2) was devised in an attempt to provide a fuller picture of the amount of radical formed by oxidation and also the amount of further oxidation of this radical to the cation. Methanol was used as the solvent which is able to act as a nucleophile to trap the cation once it is formed. The ratio of methyl ester **10** to *O*-acylhydroxylamine **9** (found by comparison of integration in ^{1}H NMR) was used to give an indication of the stability of the acyl radical with more *O*-acylhydroxylamine indicating a more stable radical.

Oxidation of hydrazides (0.4 mmol) in methanol (5 mL) with the manganese catalyst and periodic acid in the presence of one molar equivalent TEMPO provides results (Table 2) suggesting that isoniazid gives an unstable acyl radical **7b** which was easily oxidised further to the cation **8b**. However,

Table 2 Ratio of methyl ester to TEMPO adduct formed with either 5 mL methanol or three equivalents methanol in acetonitrile

Reactant	5 mL methanol	50 μL methanol in acetonitrile
Isoniazid	6 : 1	1 : 9
Benzhydrazide	0.7 : 1	1 : 8
m-Nitrobenzhydrazide	3 : 1	1 : 1
p-Methoxybenzhydrazide	4 : 1	1 : 9

the low yield of the TEMPO adduct **5** (not more than 10%) was surprising, as the yield of **5** was close to 60% under similar conditions in acetonitrile (Table 1).

To further investigate this observation, the amount of methanol was reduced to three molar equivalents with respect to the hydrazide and TEMPO, and acetonitrile was used as (non-nucleophilic) solvent. The results of these experiments are listed in Table 2. The reduction in the amount of methanol led to the formation of the TEMPO adduct as the major product for both isoniazid and benzhydrazide. This led us to propose that the intermediate diimide (**11**, Scheme 3) undergoes nucleophilic acyl substitution to yield the corresponding ester. The diimide **11** is presumably the precursor to the acyl radical **7**, therefore its interception reduces the yield of radical products.

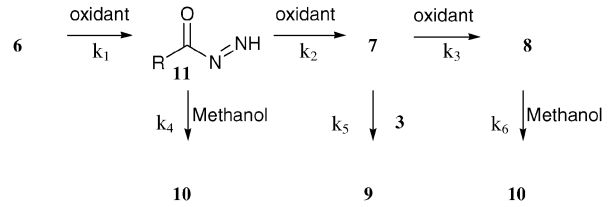
The rate of nucleophilic acyl substitution is dependent on the concentration of reactants. Where there is a large concentration of a nucleophile such as methanol, the rate of nucleophilic attack on the carbonyl carbon (Scheme 3, k_4) competes favourably with the rate of oxidation to the radical (Scheme 3, k_2). This also explains the formation of diacyl hydrazine when the concentration of oxidant is low which is a well known transformation.[6,8]

This propensity to undergo nucleophilic acyl substitution was not seen equally for all functional groups and isoniazid showed the greatest tendency to this pathway.

To maximise the production of acyl radical and further oxidation products we used the bulky alcohol isopropanol, in place of methanol. The formation of a trace of the isopropyl ester was only observed in the oxidation of *m*-nitrobenzhydrazide **6c** (Scheme 2), and in no other hydrazides. We deduced that the electron withdrawing nitro group promoted the nucleophilic substitution to a small extent in this case and that the acyl cation **8** was not formed from any of the hydrazides.

We tested this hypothesis by subjecting the hydrazides to oxidation in the presence of three equivalents of methanol with acetonitrile as solvent in the absence of TEMPO. Formation of the cation **8** (Scheme 2) would have resulted in an increased yield of methyl ester (**10** Scheme 2), however the yield of **10** in all cases remained low and instead decomposition occurred.

In order to provide further support the ionisation energies for the acyl radicals in this study were investigated using computational techniques.[14] Geometries of radicals and cations (**7** and **8a–d**, Scheme 2) were optimised using B3LYP/6-31G(d) and single point energies calculated using B3LYP/6-311 + G(2d,2p). The calculations indicated a gas phase adiabatic ionization energy from the radical to the cation (**7–8** Scheme 2) ranging from 6.3 eV to 7.3 eV (Table 3). These data were then converted into reduction potentials for the cations (**8a–d** Scheme 2) following a modification of the method of Fu *et al.* which has been shown to yield excellent agreement with experimental results.[15] Reduction



Scheme 3 Revised mechanism for the oxidation of hydrazides, proceeding through imide, radical and cation intermediates.

Table 3 Standard reduction potential of cations resulting from hydrazide oxidation calculated at B3LYP/6-311 + G(2d,2p)//B3LYP/6-31G(d)

	Gas phase ionisation energy/eV	Solvated ionisation energy/eV	E° *vs.* NHE/V
Isoniazid	7.28	4.75	0.47
Benzhydrazide	6.75	4.47	0.19
m-Nitrobenzhydrazide	7.32	4.73	0.43
p-Methoxybenzhydrazide	6.32	4.28	0.00

potentials for the acyl cations relative to the normal hydrogen electrode (NHE) were determined to lie in the range: 0.00 V to 0.47 V (Table 3). These values are substantial in comparison with a reduction potential of 0.36 V for $[Fe(CN)_6]^{3-}$[16] and support the hypothesis that the cation is not formed under the oxidative conditions described in this study.

This proposed mechanism is consistent with previous observations of hydrazide oxidation such as the oxidation with catalytic Cu^{2+} in methanol in which mostly carboxylic acids were formed. This was attributed to attack of water on the acyl cation (even with a large excess of methanol) whereas our calculations would suggest that the cation is unlikely to form and that trapping the acyl radical with oxygen would produce this result.[6a] In addition, the oxidation of hydrazides in various alcohols reported by Polanc *et al*. showed good yields for those hydrazides with electron withdrawing groups (encouraging the nucleophilic substitution pathway) but no results for electron donating groups or even benzhydrazide were reported indicating no major contribution from the cation pathway.[6b] The reaction pathway proposed can also be used to rationalise the products formed from the oxidation of isoniazid in the presence of $H_2{}^{18}O$ and $^{18}O_2$ as reported by Bernadou and Meunier.[5b]

Our results could assist in understanding why a single base pair mutation in the KatG enzyme in *Mycobacterium tuberculosis* confers resistance to isoniazid.[17] This change, from a serine to a threonine in the access channel to the heme active site,[17,18] is analagous to the change in the alcohol used for trapping from methanol to isopropanol. Our isopropanol trapping experiments yielded some additional information, as in addition to the TEMPO ester, small amounts of aldehyde (consistent with hydrogen transfer from the isopropanol to the acyl radical) were formed. The secondary alcohol moiety in the threonine side chain may be having a similar effect and may react with the intermediate radical in the access channel thus conferring resistance.

In addition, resistance to isoniazid may be caused by the mutation in KatG favouring the acyl nucleophilic substitution pathway in preference to radical formation. This competition is particularly relevant for isoniazid activation in an aqueous medium which is similar to our experiments in methanol which hindered radical formation.

In conclusion, radical trapping, and competitive trapping experiments have led to a mechanistic understanding that supports and unites all experimental data available for the oxidation of hydrazides. The oxidation of hydrazides occurs *via* a more complex mechanism than first thought, which is dependent on reaction conditions including strength of oxidant, concentration of reactants and nucleophilicity of solvents and reactants. We have also shown unequivocally that isoniazid gives an acyl radical when oxidised but the intermediate imide is susceptible to nucleophilic attack. As a result of these investigations we speculate that the resistance conferred by the single base pair change in mutant KatG could be explained by premature reaction of the acyl radical or interception of the acyl radical precursor. This information may be of benefit in the understanding of mechanisms of drug resistance with TB and also in the development of synthetic methods involving hydrazides.

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