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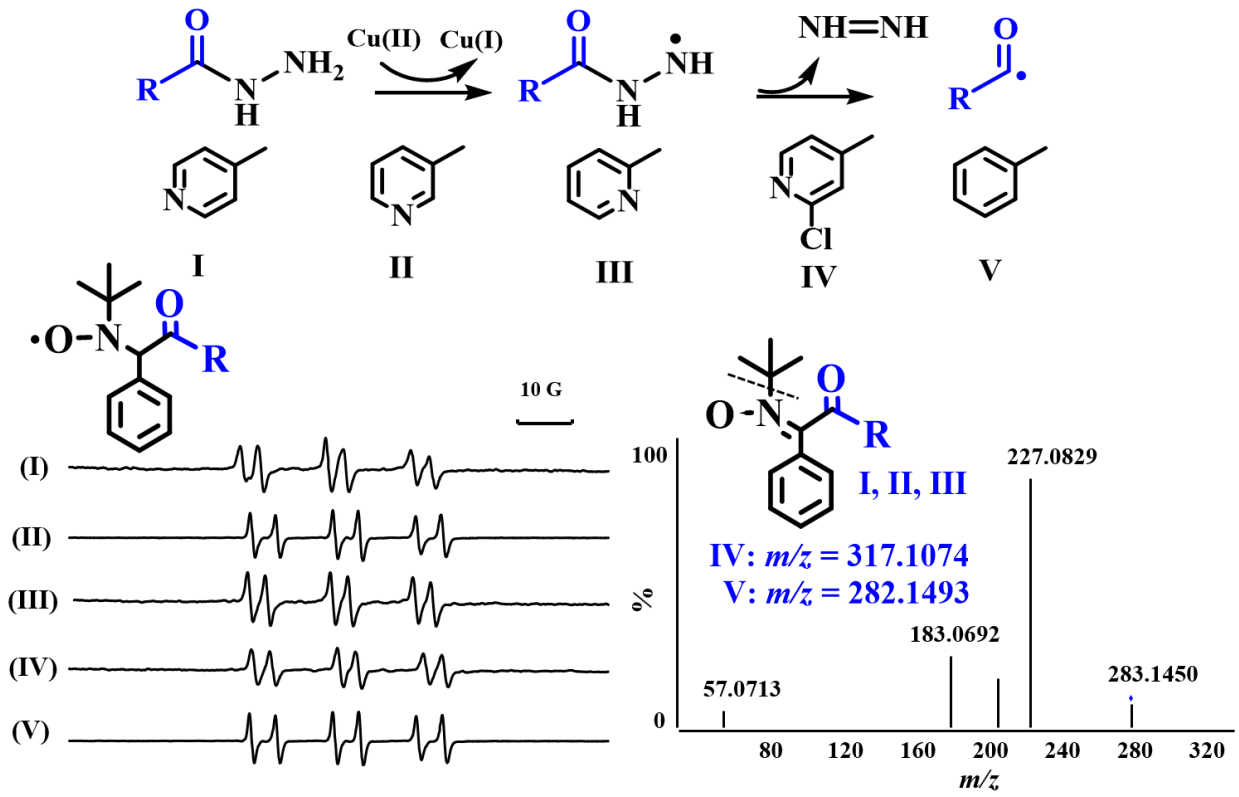

Identification of the C-centered acyl radicals by ESR spin-trapping and HPLC/MS methods

First Unequivocal Identification of the Critical Acyl Radicals from the Anti-Tuberculosis Drug Isoniazid and its Hydrazide Analogs by Complementary Applications of ESR Spin-trapping and HPLC/MS Methods

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Abstract: The carbon-centered isonicotinic acyl radical of isoniazid (INH), a widely-used frontline anti-tuberculosis drug, has been considered to play a critical role in inhibiting *Mycobacterium tuberculosis*, but not fully identified. Here we show that this radical intermediate can be unequivocally characterized by complementary applications of ESR spin-trapping and HPLC/MS methods by employing *N-tert*-butyl-α-phenylnitrone (PBN) as the suitable spin-trapping agent, which can form the most stable radical adduct. More importantly, for the first time, analogous carbon-centered acyl radicals and their respective NAD^+ adducts have also been detected and identified from its two isomers (nicotinic acid hydrazide and 2-pyridinecarbohydrazide) and benzhydrazide which are structurally-related to INH, but not by 2-chloroisonicotinohydrazide. This study represents the first unequivocal identification of the carbon-centered acyl radicals of INH and other hydrazide analogs by both ESR spin-trapping and HPLC/MS methods, which may have broad biomedical and toxicological significance for future research for more efficient hydrazide anti-tuberculosis drugs.

Keywords: Isoniazid, Hydrazides, Carbon-centered acyl radicals, ESR spin-trapping/HPLC/MS, *N-tert*-butyl-α-phenylnitrone (PBN), NAD^+ adducts

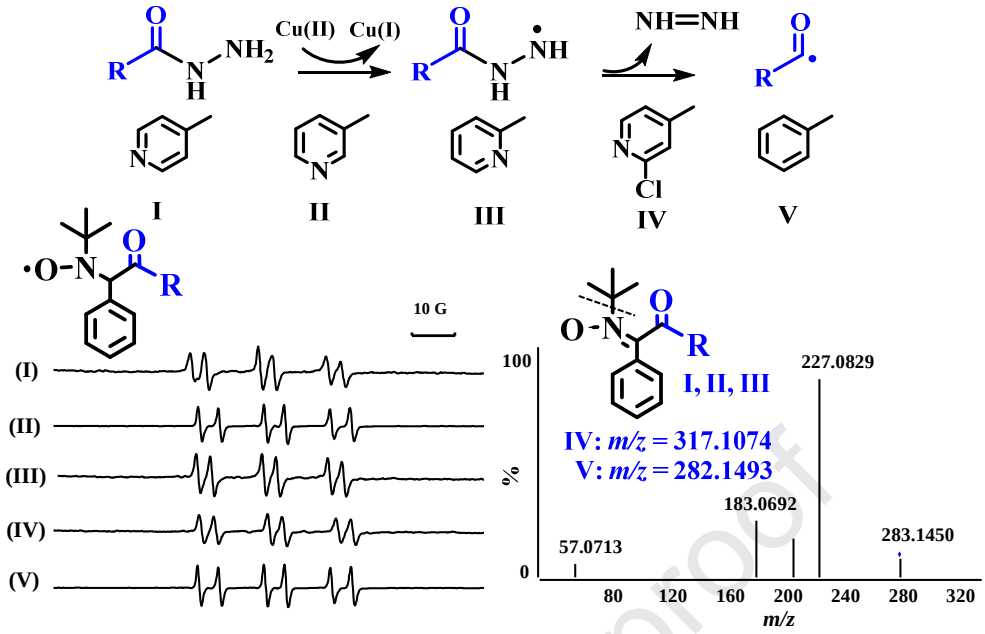
Research Highlights

► PBN was the best spin-trapping agent to trap the C-centered radical of INH

► C-centered radicals of hydrazides were identified by ESR and HPLC/MS methods

► NAD^+ adducts with C-centered radicals were detected by HPLC/MS except for Cl-INH

Graphical Abstract



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Introduction

As one of the hydrazine derivatives, isoniazid (INH) was invented in 1952. INH is considered as one of the most effective inhibitors of *Mycobacterium tuberculosis* and widely used frontline anti-tuberculosis drugs for almost 70 years [1,2]. Tuberculosis is still a major health problem and a cause of death in undeveloped and developing countries[3]. It has been shown that INH could be activated by catalase-peroxidase KatG to isonicotinic acyl radical ($RCO^{\bullet}$) which combined nicotinamide adenine dinucleotide (NAD^{+}) to form an INH-NAD adduct to inhibit InhA[1,4,5]. As an encol-acyl carrier protein, InhA inhibition could result in inhibiting the fatty acid synthesis pathway of *Mycobacterium tuberculosis*, thus preventing the production of mycolic acid required for cell wall synthesis[1,2,6]. Therefore, it was considered that the oxidation of INH and generation of isonicotinic acyl radical may play an important role in this process.

The activation of INH was postulated to involve free radical intermediates and this hypothesis was supported by ESR spin-trapping method [7-10]. Previous studies have shown that a C-centered radical can be detected by ESR using 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) [9,11] and *N*-*tert*-butyl-α-phenylnitrone (PBN) as the trapping agent [8,12-15]. However, it remained not fully clear whether the detected C-centered radical was isonicotinic acyl radical in these ESR spin-trapping studies.

Although the C-centered radical of INH could be observed by ESR spin-trapping method, little information is available on the C-centered radicals from other hydrazides, including the structural isomers of INH (nicotinic acid hydrazide (NH) and 2-pyridinecarbohydrazide (2-NH)), benzhydrazide (BH) and 2-chloroisonicotinohydrazide (Cl-INH).

Therefore, in this study, we addressed the following questions: (*i*) whether the previously-proposed C-centered isonicotinic acyl radical intermediate can be detected and unequivocally identified during INH oxidation by complementary applications of ESR spin-trapping and HPLC/MS methods; (*ii*) whether similar *C*-centered acyl radical could be formed from the isomers of INH and other hydrazides by ESR using

different spin-trapping agents and HPLC/MS; if so, what is the structure-activity relationship? (*iii*) what are the potential biological and environmental implications of our new findings?

Experimental Section

Materials

Isoniazid (INH), 2-pyridinecarbohydrazide (2-NH), nicotinic acid hydrazide (NH), 2-chloroisonicotinohydrazide (Cl-INH), benzhydrazide (BH), nicotinamide adenine dinucleotide (NAD^+), 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO), *N*-*tert*-butyl-α-(4-pyridyl-1-oxide)nitrone (POBN), *N*-*tert*-butyl-*α*-phenylnitrone (PBN), MnO_2 and $CuSO_4$ were purchased from Sigma (St. Louis, MO). 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and 5-*tert*-butoxycarbonyl-5-methyl-1-pyrroline *N*-oxide (BMPO) were purchased from DOJINDO MOLECULAR TECHNOLOGIES, INC. The chemical structures of the hydrazides and spin-trapping agents were shown in Fig. S1. All buffer solutions were treated with Chelex for 24 h to remove adventitious metals[16].

ESR studies

The free radicals were recorded on Bruker ESR 300 spectrometer at room temperature during the reaction of INH and other hydrazides with Cu(II) and MnO_2. The trapping agent includes DMPO (100 mM). Typical spectrometer parameters were as follows: scan range, 100 G; Center field, 3511 G; time constant, 300 ms; scan time, 100 s; modulation amplitude, 1 G; modulation frequency, 100 kHz; receiver gain, 1.25×10^5; and microwave power, 20 mW and number of X-Scans, 10.

HPLC-ESI-Q-TOF-MS analysis

The C-centered radicals of hydrazides with PBN, TEMPO and NAD^+ adducts were analyzed by high-performance liquid chromatography combined with electrospray ionization quadruple time-of-flight mass spectrometry (HPLC-ESI-Q-TOF-MS). The adducts were analyzed on the 4.6 × 250 mm reversed-phase C18 analytical columns with 5 μm silica-based (Waters XTERRA® MS). The PBN/C-centered acyl radical adducts were eluted with 70% formic acid (0.1%) and 30% acetonitrile. The

TEMPO/C-centered acyl radical adducts were eluted with 40% formic acid (0.1%) and 60% acetonitrile. The NAD^+/C-centered acyl radical adducts were eluted with 90% formic acid (0.1%) and 10% methanol. MS and MS/MS spectra were acquired in positive-ion mode. The operating parameters were as follows: capillary voltages 2.5 kV, sample cone voltages 30 V, source temperature 80 °C, desolvation temperature 200 °C. Nitrogen (99.99%) was used as the drying gas. The collision gas was argon at the pressure of 5.0×10^{-5} Torr, and the collision energy was 10 V.

Oxygen Consumption

The O_2 consumption was monitored with an Orion-type oxygen electrode (Thermo Electron Corporation) upon mixing hydrazides with or without Cu(II)/Mn(III) in phosphate buffer (100 mM, pH 7.4).

Results and Discussion

Cu(II) was found to be the best to activate INH to produce C-centered radical as detected by ESR spin-trapping

It has been shown that C-centered isonicotinic acyl radical formation is critical in the process of INH oxidation to inhibit InhA which is essential for *Mycobacterium tuberculosis* [1,4,17,18]. At the beginning of this study, we tried to detect the C-centered isonicotinic acyl radical by ESR spin trapping technique with DMPO as the spin-trapping agent by using several different oxidants to activate INH, including Cu(II)SO_4, Mn(III)-pyrophosphate, MnO_2, horseradish peroxidase, and myeloperoxidase. Among them, Cu(II) was found to be the best to activate INH to produce C-centered radical (Fig. S2, S3). Cu(II) was found to be an essential trace element in organisms [19,20]. As a redox-active transition metal, Cu(II) comprised the active center of a wide variety of metalloenzymes, such as Cu, Zn superoxide dismutase and cytochrome c oxidase [19,20]. However, it has also been reported that Cu(II) can induce oxidative damage to DNA, proteins, lipids in both chemical and cell culture systems , thereby potentially contributing to disease pathology [21-33]. These damages induced by Cu(II) might result from its redox properties, in particular its

participation in a copper-catalyzed, Fenton-like reaction [23,24,34]. We speculated that the radicals generated from INH and Cu(II) should contain at least two types of radical species: a 9-line hydrogen radical (a^{H1} = a^{H2} = 22.69 G, a^{N} = 16.35 G) and a 6-line C-centered radical (a^{H} = 22.92 G, a^{N} = 15.62 G). The formation of hydrogen radical was further supported by the control result (hydrazine/Cu(II))[35,36] and the simulation results (Fig. S3c-d). The formation of C-centered radical was also further confirmed by the simulation results (Fig. S3e). It was speculated that the hydrogen radical might be generated via one of the decomposition pathways of the N-centered radical $RCONHNH^{\bullet}$ which was recently identified by our research group [37].

$$RCONHNH^{\bullet} \longrightarrow RCON{=}NH + H^{\bullet}$$

According to previous ESR studies, it was speculated that the C-centered radical was isonicotinic acyl radical, which is a key intermediate to form INH-NAD^{+} adduct in the presence of NAD^{+}.

The formation and decay of the C-centered radical of INH was found to be dependent on the concentrations of both INH and Cu(II), and the molar ratios of INH/Cu(II) (Fig. S4a-b). As shown in Fig. S4a, when using DMPO as the spin-trapping agent, with a fixed Cu(II) concentration (0.5 mM), the intensity of the C-centered radical increased with the increasing of INH concentration, however, it decayed faster at higher INH concentration. The time course of the formation of C-centered radical was relatively high and stable at the molar ratio of 2:1 (INH/Cu). Moreover, with a fixed INH concentration, the C-centered radical decayed slower with the increasing of Cu(II) concentration (Fig. S4b). The reason might be that Cu(II) was precipitated at higher concentration and the reaction rate was decreased.

PBN was found to be the best to trap the C-centered radical from INH among the four spin trapping agents tested

The above results showed that C-centered isonicotinic acyl radical could be detected by using DMPO as the spin-trapping agent, however, the DMPO-isonicotinic acyl radical adduct was found to be not stable enough for further characterization by

HPLC/MS analysis. To find a more stable radical adduct, other spin-trapping agents including BMPO, POBN and PBN have also been tested. As shown in Fig. 1, carbon-centered radical of INH could be detected by all three other spin-trapping agents. Among them, PBN was found to be the best to trap the isonicotinic acyl radical (Fig. 1).

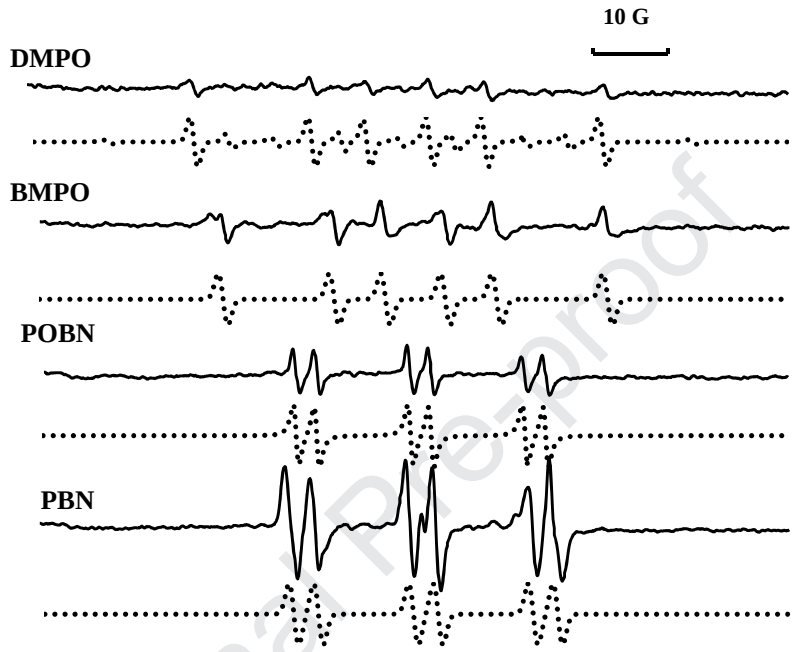


Fig. 1. ESR spectra of C-centered radical produced by incubation of INH with Cu(II) in the presence of four spin-trapping agents (DMPO, BMPO, POBN and PBN). (a): ESR spectra of DMPO spin trapping adducts with C-centered radical and the simulation spectra (a^H = 22.92 G, a^N = 15.62 G); (b) ESR spectra of BMPO spin trapping adducts with C-centered radical and the simulation spectra (a^H = 21.65 G, a^N = 14.81 G); (c): ESR spectra of POBN spin trapping adducts with C-centered radical and the simulation spectra (a^H = 2.77 G, a^N = 15.33 G); (d): ESR spectra of PBN spin trapping adducts with C-centered radical and the simulation spectra (a^H = 3.31 G, a^N = 16.06 G). Reactions contained the spin-trapping agents (DMPO, BMPO, POBN and PBN) 100 mM, 6 mM INH and 0.5 mM Cu(II). All reactions were conducted in Chelex-treated phosphate buffer (100 mM, pH 7.4). The range of the ordinate value was (-10~10)*10^6.

Interestingly, we also found that PBN/C-centered radical was much more stable than DMPO/C-centered radical (Fig. S4). The half-life of DMPO/C-centered radical was found to be about 20 min, while the half-life of PBN/C-centered radical is about 24 h! The formation and decay of the PBN/C-centered radical of INH was also found to be dependent on the concentration of both INH and Cu(II), and the molar ratios of

INH/Cu(II) (Fig. S4c-d). These results suggested that the spin-trapping agent PBN was better than DMPO to trap this C-centered radical.

The C-centered radical trapped by PBN was further unequivocally identified as isonicotinic acyl radical by HPLC/MS

To further characterize PBN/C-centered radical adduct, we have used HPLC combined with electrospray ionization quadrupole time-of-flight mass spectrometry (HPLC-ESI-Q-TOF-MS). Although we can observe the ESR signal for nitroxide radical form of DMPO/isonicotinic acyl radical, we cannot directly detect its corresponding nitrone form by HPLC/MS. Interestingly, a new peak with the retention time at 4.40 min was observed from the reaction between INH and Cu(II) in the presence of PBN by HPLC-ESI-Q-TOF-MS in positive mode (Fig. 2a). This new compound was characterized by ESI-Q-TOF-MS at *m/z* 283 (Fig. 2b), indicating that the new compound should be the deprotonated PBN/isonicotinic acyl radical nitrone adduct, which was further confirmed by MS/MS study (ESI-positive) (Fig. 2c). As shown in Fig. 1, a typical 6-line ESR signal was also observed in the reaction of INH and Cu(II) with PBN. The above studies by both ESR using PBN as spin-trapping agent and HPLC/MS methods unequivocally confirmed that the C-centered radical was indeed the isonicotinic acyl radical.

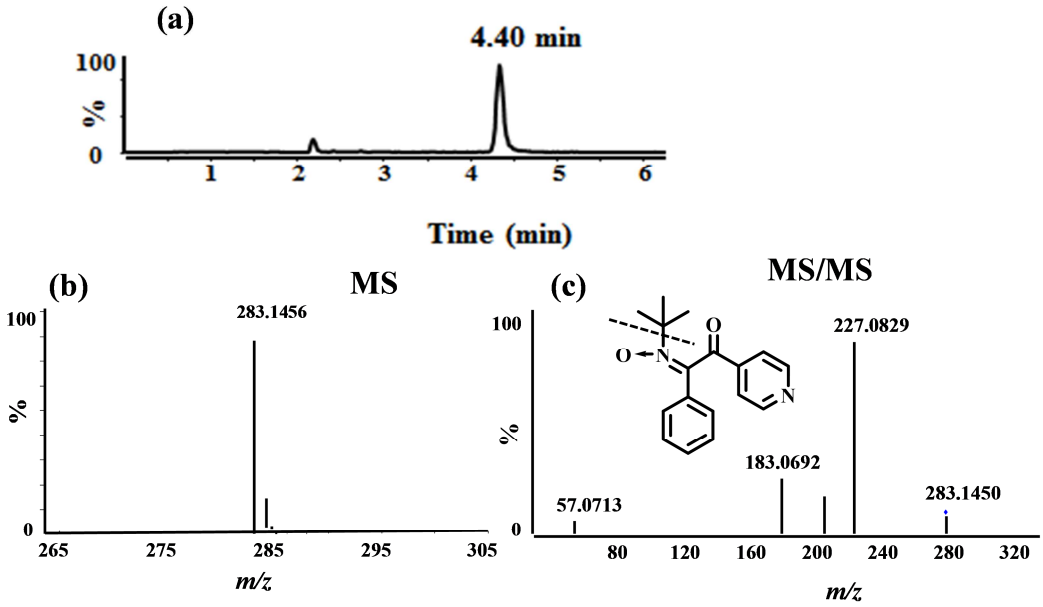


Fig. 2. Unequivocal identification by HPLC/MS of PBN/isonicotinic acyl radical derived nitrone adduct from INH/Cu(II) in the presence of PBN. (a): The HPLC/MS selected ion monitoring (SIM) profile of the reaction mixtures of 2 mM INH and 0.5 mM Cu(II); (b): The ESI-Q-TOF-MS spectra of deprotonated PBN/isonicotinic acyl radical nitrone adduct with a retention time of 4.40 min; (c): MS/MS spectrum of the ion peak at *m/z* 283. Reactions were carried out at room temperature in Chelex-treated phosphate buffer (100 mM, pH 7.4).

Analogous C-centered radicals were found to be produced by two INH isomers and other hydrazides

To test whether the formation of C-centered radical is a general mechanism for the activation of other hydrazides by Cu(II) using DMPO as the trapping agent, we have studied four other hydrazides which are structurally related to INH. These include its two isomers (nicotinic acid hydrazide (NH), and 2-pyridinecarbohydrazide (2-NH)), its chlorine-substituted derivative 2-chloroisonicotinohydrazide (Cl-INH) and benzhydrazide (BH). Except for 2-NH, ESR spectra of DMPO/C-centered radicals were readily observed from these hydrazides activated by Cu(II) (Fig. 3b-c). Interestingly and unexpectedly, C-centered radical could not be observed from 2-NH when oxidized by Cu(II), even with other different spin-trapping agents (BMPO, PBN, POBN) (Fig. S5a). We speculated that this might be because that there might be special interaction between Cu(II) and 2-NH. Indeed we found that Cu(II) is most effective in accelerating the oxidation of 2-NH among the five hydrazides as measured by oxygen consumption (Fig S6a), and a new complex with the maximal absorption peak at 360 nm was formed as shown by UV–Vis spectra studies (Fig. S7). In the reaction of 2-NH with Cu(II), a new product 1,2-dipicolinoylhydrazine was detected and identified by HPLC/MS (Fig.S8), which was found to be similar with that of INH [37]. Therefore, we speculated that the absorption peak at 360 nm was the complex of the new final reaction product 1,2-dipicolinoylhydrazine with Cu(II) (for more details, see SI).

To enable 2-NH to produce C-centered radical, we tried several other oxidants other than Cu(II). When Mn(III)-pyrophosphate was used, C-centered radical was observed from 2-NH only when using POBN and PBN, but not DMPO and BMPO, as

spin-trapping agents. More interestingly, the formation of *N*-centered radical intermediate could also be readily observed from 2-NH/Mn(III)-pyrophosphate with DMPO and BMPO (Fig. S5b)[37]. In contrast, when MnO_2 was employed as the oxidizing agent, C-centered radical could be readily observed from 2-NH with DMPO (Fig. 3c). For the other four hydrazides (INH, NH, Cl-INH and BH), C-centered radicals could also be detected by employing MnO_2 as the oxidizing agent (Fig. S10), however, the detected ESR signals for C-centered radicals were found to be weak for INH and Cl-INH (Fig. S10). These results indicated that the production of C-centered radical was a general phenomenon for all hydrazides tested when activated by suitable oxidizing agents.

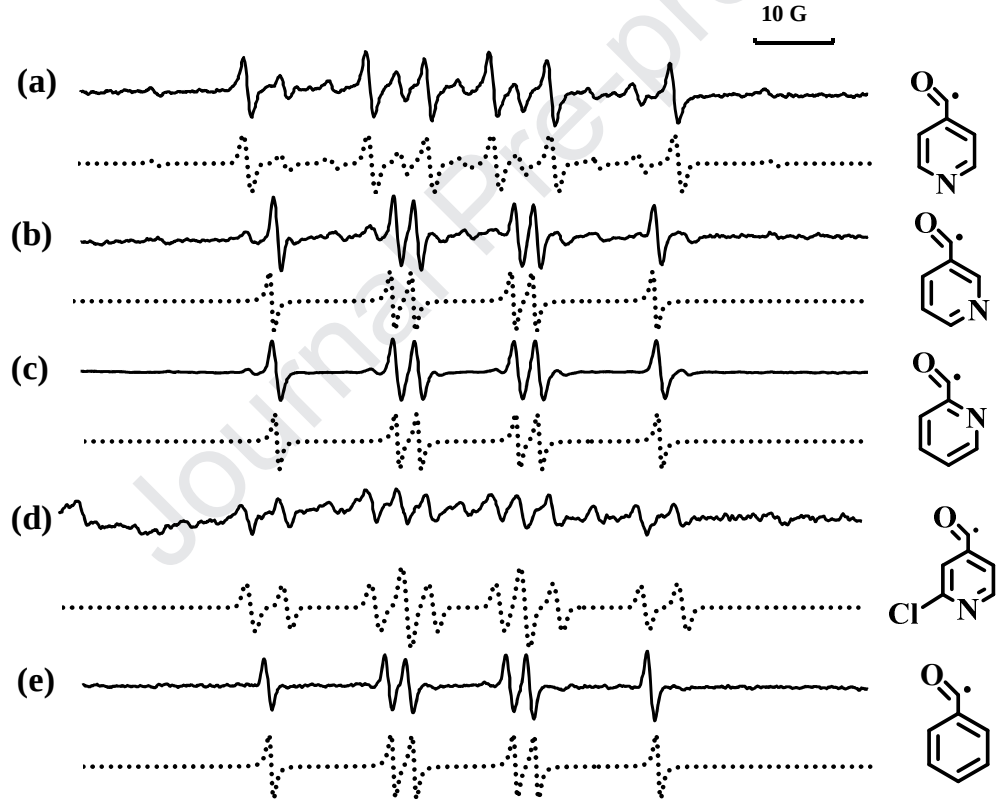
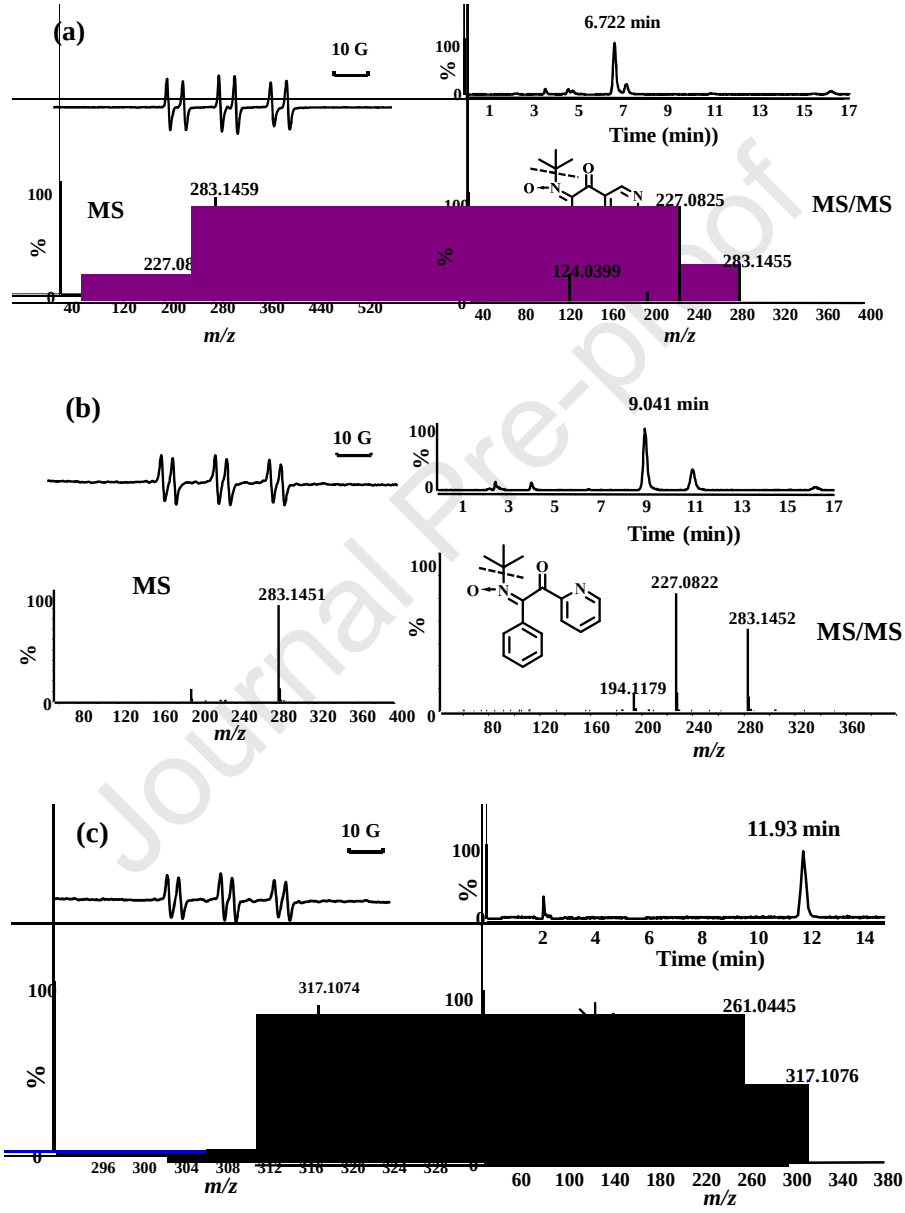


Fig. 3. ESR spectra of free radical intermediates produced by incubation of INH and four other hydrazides with Cu(II) or MnO_2 in the presence of DMPO. (a): Reaction mixtures contained 100 mM DMPO, 6 mM INH and 0.5 mM Cu(II), a^H = 22.92 G, a^N = 15.62 G; (b): Reaction mixtures contained 100 mM DMPO, 6 mM NH and 0.5 mM Cu(II), a^H = 17.75 G, a^N = 15.28 G; (c): Reaction mixtures contained 100 mM DMPO, 6 mM 2-NH and MnO_2, a^H = 18.06 G, a^N = 15.35 G; (d): Reaction mixtures contained 100 mM DMPO, 6 mM Cl-INH and 0.5 mM Cu(II), C-centered radical a^H = 23.00 G, a^N = 15.52 G, and hydroxyl radical a^H = a^N = 14.92 G; (e): Reaction mixtures contained 100 mM DMPO, 6 mM BH and 0.5 mM Cu(II), a^H =

17.94 G, a^N = 15.36 G. The reaction was conducted in Chelex-treated phosphate buffer (100 mM, pH 7.4).

The C-centered radicals produced from other hydrazides were identified as their corresponding acyl radicals by complementary applications of ESR and HPLC/MS by using PBN as the spin-trapping agent



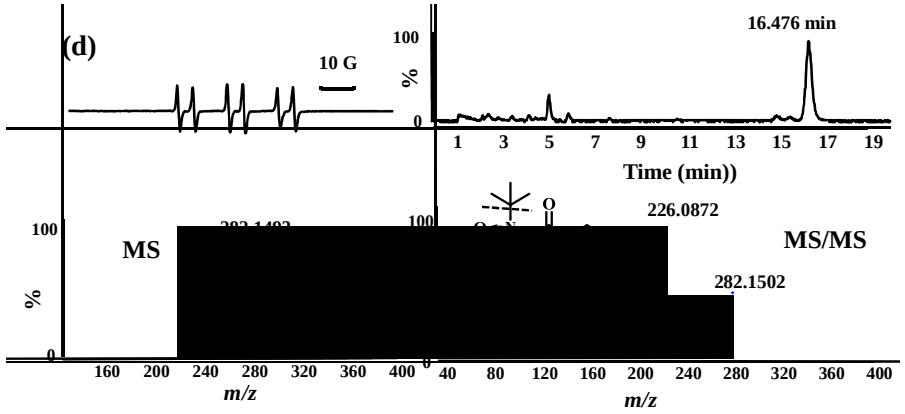


Fig. 4. Unequivocal identification by both ESR spin-trapping and HPLC/MS of PBN/C-centered acyl radical derived nitrone adduct from the other four hydrazides in the presence of PBN. (a): Identification of PBN/C-centered radical derived nitrone adduct produced by NH/Cu(II), a^H = 4.7 G, a^N = 15.24 G; (b): Identification of PBN/C-centered radical derived nitrone adduct produced by 2-NH/MnO_2, a^H = 4.07 G, a^N = 15.85 G; (c): Identification of PBN/C-centered radical derived nitrone adduct produced by Cl-INH/Cu(II), a^H = 3.45 G, a^N = 15.86 G; (d): Identification of PBN/C-centered radical derived nitrone adduct produced by BH/Cu(II), a^H = 4.8 G, a^N = 15.44 G. Reactions were carried out at room temperature in Chelex-treated phosphate buffer (100 mM, pH 7.4).

The above ESR spin-trapping studies can only suggest that what we detected are C-centered radicals derived from hydrazides, it was not clear whether they are the C-centered acyl radicals similar to that produced from INH. To address this question, HPLC-ESI-Q-TOF-MS was also employed to further identify the exact composition of PBN/C-centered radical adduct. As shown in Fig. 4, six-line ESR spectra were obtained by all of the four hydrazides (NH, 2-NH, Cl-INH and BH) in the presence of PBN. As expected, new peaks with *m/z* 283 and the retention time of 6.722 and 9.041 min, respectively, were observed with NH/Cu(II) and 2-NH/MnO_2 (Fig. 4a-b). For Cl-INH, a new peak with one-Cl isotope clusters at 11.93 min with *m/z* 317 was observed from Cl-INH and Cu(II) (Fig. 4c). For BH, a new peak with *m/z* 282 and the retention time at 16.476 min was observed from BH and Cu(II) (Fig. 4d). The MS/MS studies (ESI-positive) further confirmed that these new compounds were the deprotonated PBN/C-centered acyl radical nitrone adducts. Taken together, the C-centered radicals produced from other hydrazides were identified as their corresponding acyl radicals by complementary applications of both ESR and

HPLC/MS by using PBN as the spin-trapping agent.

The formation of C-centered acyl radicals were further confirmed by nitroxide radical trapping agent TEMPO

In addition to PBN, TEMPO, as a persistent nitroxide radical, was also used as an effective radical trapping agent [38]. The TEMPO adduct of C-centered isonicotinic acyl radical of INH has been detected and identified in previous studies [2,39]. In this study, the TEMPO adducts of C-centered acyl radicals derived from the other four hydrazides (NH, 2-NH, Cl-INH and BH) have also been studied. We found that all of the C-centered acyl radicals could combine with TEMPO to generate their corresponding TEMPO adducts (Fig. S11). For INH, NH and 2-NH, the new peaks with *m/z* 263 were observed at the retention time of 14.890, 6.810 and 15.312 min, respectively (Fig. S11a-c). For Cl-INH, a new peak with one-Cl isotope clusters with *m/z* 297 at 15.334 min was observed from Cl-INH and Cu(II) (Fig. S11d). For BH, a new peak with *m/z* 262 and the retention time at 17.239 min was observed from BH and Cu(II) (Fig. S11e). These results were further confirmed by MS/MS studies.

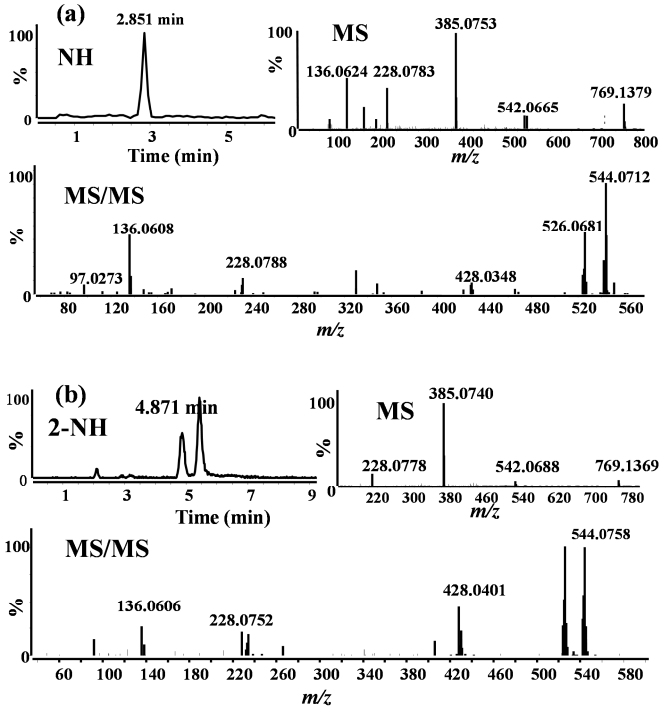
In addition to detecting the TEMPO/C-centered radicals adducts by HPLC/MS, we have also compared the relative yield of them derived from the five hydrazides (the extracted peak area) (Fig. S12). We found that the amount of C-centered radical derived from BH was the most when using Cu(II) as the oxidant, while the amount of C-centered radical derived from 2-NH was the least (Fig. S12a-b). When using Mn(III) as the oxidant, we found that the amount of C-centered radical derived from BH was still the most, while C-centered radical derived from Cl-INH was the least (Fig. S12c-d).

Detection and identification of NAD^+ adducts with C-centered acyl radicals produced by the four other hydrazides

It has been shown that INH could be activated to form isonicotinoyl acyl radical, which can react with NAD^+ to form an acyl-NAD^+ adduct, leading to inhibition of *M. tuberculosis*[1,4,40]. Therefore, it will be interesting to check whether acyl radicals

generated from the other four hydrazides (NH, 2-NH, Cl-INH and BH) could also react with NAD^+ to form analogous adducts. To address this question, HPLC-ESI-Q-TOF-MS was employed to further identify the acyl-NAD^+ adducts. To our delight, we found that the C-centered acyl radicals generated from NH, 2-NH and BH could also combine with NAD^+ to form their respective adducts (Fig. 5). For NH and 2-NH, the new peaks with *m/z* 769 were observed at the retention time of 2.851 and 4.871 min, respectively (Fig. 5a-b). For BH, the new peak with *m/z* 768 was observed at the retention time of 13.100 min (Fig. 5c).

Interestingly, for Cl-INH, no NAD^+ adduct was detected under our experimental conditions. Since it was suggested that the adduct isonicotinic acyl-NAD^+ was formed through the addition of either an isonicotinic acyl anion to NAD^+ or an isonicotinic acyl radical to an $NAD^{\bullet}$ radical[1], we speculated that the electron withdrawing chlorine group may decrease the ability of the formation of C-centered aycl radical or an acyl anion from Cl-INH [2]. In agreement with this finding, the ESR spin-trapping results also showed that Cl-INH produced the least amount of C-centered acyl radical as compared to the other four hydrazides (Fig. S12). Therefore, the NAD^+ adduct with the C-centered acyl radical generated from Cl-INH might be too little to be detected.



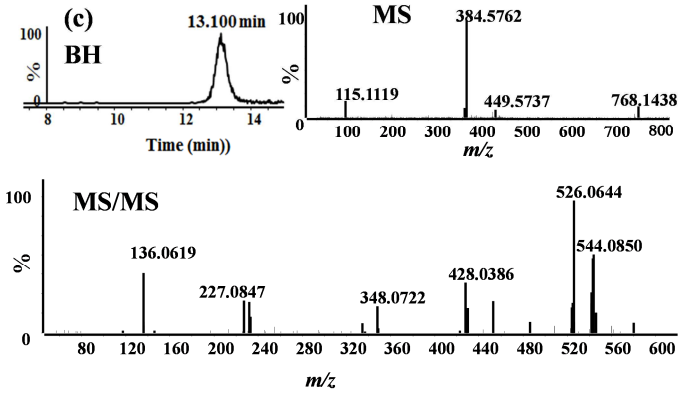


Fig. 5. Identification of adducts of NAD$^+$ with C-centered radical of NH, 2-NH and BH by using HPLC-ESI-Q-TOF-MS. (a): The retention time, MS and MS/MS spectra of NH-NAD$^+$; (b): The retention time, MS and MS/MS spectra of 2-NH-NAD$^+$; (c): The retention time, MS and MS/MS spectra of BH-NAD$^+$.

Potential biological and toxicological implications

The carbon-centered isonicotinic acyl radical of isoniazid (INH), a widely-used frontline anti-tuberculosis drug, has been considered to play a critical role in inhibiting *Mycobacterium tuberculosis*, but not fully identified. Previous ESR spin-trapping studies can only suggest that what they detected were C-centered radical derived from INH, it was not certain whether it was the C-centered acyl radicals produced from INH. To address this question, we employed both ESR and HPLC-ESI-Q-TOF-MS methods which we have often used by our research group to further identify the exact composition of the trapping agent/C-centered radical adduct [41-56]

Through comparison of four widely-used spin-trapping agents, we found PBN can trap C-centered isonicotinic acyl radical to form the most stable nitroxide radical form of PBN/C-centered isonicotinic acyl radical adduct, which is stable enough for HPLC/MS study. Based on this finding, we have unequivocally identified the C-centered radical is indeed the acyl radical of INH by both ESR and HPLC/MS methods by employing PBN as a suitable spin-trapping agent.

More interestingly, we found that the formation of the C-centered acyl radicals was not only limited to INH, but it was also a general mechanism for the activation of other four hydrazides which have similar chemical structures. In addition to the

detection of the PBN/C-centered acyl radicals nitrone adducts, the TEMPO adducts with all of the other four hydrazides (NH, 2-NH, Cl-INH and BH) have unequivocally identified by HPLC/MS. More importantly, the NAD^+ adducts with C-centered acyl radicals derived from NH, 2-NH and BH, but not from Cl-INH, have also been detected by HPLC/MS. It is worth noting that our results is in good agreement with a previous study, which has been shown that substitution of withdrawing groups at the *ortho* position to the ring nitrogen yields compounds with decreased antibacterial action, which was attributed to the change of the pK_a value and steric effect [57]. It should be noted, however, although the amount of C-centered radicals derived from NH and BH were much higher than that of INH (Fig. S10) and NAD^+ adducts of NH and BH could also be detected in our study, it has been shown that their antibacterial activities against *M. tuberculosis* were weaker than that from INH [8,57,58]. The reason might be that, beside the formation of C-centered radicals, other factors such as the differences in electronic effects and hydrophobic properties may also play some roles [57,59]. Therefore, our findings may have potential biomedical and toxicological implications.

To our knowledge, this is the first detection and unequivocal identification of C-centered acyl radicals derived from INH, its isomers and other hydrazides by both ESR trapping and HPLC-MS methods. The identification of the C-centered acyl radicals of hydrazides may be of benefit in understanding of molecular mechanisms of activation of INH and other hydrazides to form the reactive radical intermediates. Moreover, the identification of the C-centered acyl radicals of hydrazides can also help explain the mechanism for the formation of their oxidation products.

Interestingly, our structure-reactivity relationship investigations found that the formation and properties of the C-centered acyl radicals intermediate were also dependent on the chemical structures of hydrazides. The C-centered acyl radicals could be generated from all of five hydrazides, however, there are also some clear differences. For NH and BH, their NAD^+ adducts with the C-centered radicals could be detected and identified. However, it has been reported that NH and BH have higher MIC than INH and their antibacterial activities against *M. tuberculosis* were weaker

than that of INH [8,57,58,60]. We also found that the C-centered radical of 2-NH could be readily detected when activated by MnO_2, but not by Cu(II), which might be due to formation of an unstable Cu(II)/2-NH complex, resulting in rapid intra-molecular electron transfer reactions. While for Cl-INH, the amount of the C-centered radicals of Cl-INH was the least and the NAD^+ adduct with C-centered radical could not be detected, which might be due to the influence of the electron withdrawing chlorine group [2]. Therefore, it was supposed that the formation of the reactive radical species could be fine-tuned by structural design, which may provide new leads in the development of more effective anti-tuberculosis drugs.

Based on the new findings in this study, it will be very tempting to further investigate the following questions in the future: 1) Whether the three new NAD^+-adducts can inhibit InhA enzyme activity, and if so, which one is more effective? 2) Whether Cl-INH is active or not in killing *Mycobacterium tuberculosis?* 3) Whether an INH derivative with an electron donating group is more effective in producing NAD^+ adduct and killing *Mycobacterium tuberculosis?*

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► PBN was the best spin-trapping agent to trap the C-centered radical of INH

► C-centered radicals of hydrazides were identified by ESR and HPLC/MS methods

► NAD^+ adducts with C-centered radicals were detected by HPLC/MS except for Cl-INH