^{1}H and ^{13}C NMR Characterization of Hemiamidal Isoniazid-NAD(H) Adducts as Possible Inhibitors Of InhA Reductase of *Mycobacterium tuberculosis*

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Abstract: Isoniazid (INH) is easily oxidized with manganese(III) pyrophosphate, a chemical model of the KatG protein involved in activation of INH inside the bacteria *Mycobacterium tuberculosis*. Performed in the presence of NAD^{+}, this oxidation generates a family of isomeric INH–NAD(H) adducts, which have been shown to be effective inhibitors of InhA, an enzyme essential in mycolic acid biosynthesis. In this work, we fully characterized by ^{1}H and ^{13}C NMR spectroscopy four main species of INH–NAD(H) adducts that coexist in solution. Two of them are open diastereoisomers consisting of the covalent attachment of the isonicotinoyl radical at position four of the nicotinamide coenzyme. The other two result from a cyclization involving the amide group from the nicotinamide and the carbonyl group from the isonicotinoyl radical to give diastereoisomeric hemiamidals. Although an INH–NAD(H) adduct with a 4*S* configuration has been characterized within the active site of InhA from X-ray crystallography and this bound adduct interpreted as an open form (Rozwarski et al., Science **1998**, *279*, 98–102), it is legitimate to raise the question about the effective active form(s), open or cyclic, of INH–NAD(H) adduct(s). Is there a single active form or are several forms able to inhibit the InhA activity with different levels of inhibitory potency?

Keywords: InhA · inhibitors · isoniazid · NAD(H) adducts · oxidation

Introduction

Isoniazid (isonicotinic acid hydrazide, or INH) is one of the oldest synthetic antituberculosis drugs that has been and is still widely used in prophylaxis and treatment of tuberculosis.[1] It is a prodrug[2] activated by the mycobacterial KatG catalase–peroxidase to generate an active form, most likely the isonicotinoyl radical, responsible for the lethal effect on bacterial cells through covalent addition on the nicotinamide moiety of NAD(H).[3, 4] This coenzyme is the cofactor of the InhA reductase,[5] a key enzyme involved in the mycolic acids synthesis.[6] In solution, UV absorption and mass spectral data of crude mixtures of INH–NAD(H) adducts, formed either by KatG-dependent processes or by nonenzymatic O_2/Mn^{II} activation, have been reported[4, 7] and shown to be competitive inhibitors of InhA.[3] A single isonicotinoyl–NAD(H) adduct has been characterized within the active site of InhA from X-ray crystallography, and this bound adduct was interpreted as the open form **2** with a 4*S* configuration (Figure 1).[3] In contrast, on the basis of mass analyses and by using manganese(III) pyrophosphate as INH activation system (to mimic the mycobacterial KatG enzyme) in the presence of NAD^{+}, we recently proposed that six open or cyclized adducts with a dihydropyridine structure are concomitantly formed in solution (compounds **1**–**6** in Figure 1).[8] Up to now, no sufficient knowledge on the stability of

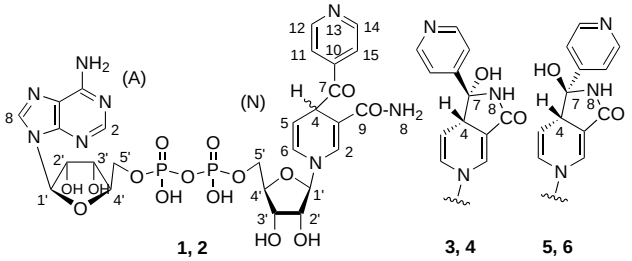

Figure 1. Structures of the covalent adducts isoniazid-NAD(H). Compounds **1** and **2** are the two diastereoisomers 4*R* and 4*S*. Compounds **3** and **4** are the two diastereoisomers 4*S*,7*R* (shown) and 4*R*,7*S* (not shown), respectively; these attributions were done on the basis of biological activity[9] and of NMR analyses of mixtures enriched in one of the two diastereoisomers. Compounds **5** and **6** are the two diastereoisomers 4*S*,7*S* (shown) and 4*R*,7*R* (not shown); these two compounds were not detected by NMR spectroscopy (this study), but were previously detected by LC-MS analyses.[8]

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these adducts and on their possible interconversion is available to allow both a complete characterization on pure species and evaluation of their own potency as InhA inhibitors. However, since preliminary results obtained with some isolated species present in solution (see reference [4] and our recent study[9]) indicate different level of activity, it is legitimate to raise the question about the exact structure of the effective active form(s) of the inhibitor.[9] Here, using a purified pool of INH–NAD(H) adducts, we report the full characterization by ^{1}H and ^{13}C NMR spectroscopy of the four main adducts formed in solution when NAD^+ was treated with INH activated by manganese(III) pyrophosphate. Besides the two open structures **1** and **2**, two cyclized adducts (**3** and **4**) with an hemiamidal structure were evidenced. Traces of the adducts **5** and **6** were previously detected by LC-MS,[8] but are present in too low an amount for NMR analysis.

Results and Discussion

Since INH–NAD(H) adducts are easily interconverted and degradated depending on the pH, ionic strength, and medium composition, NMR analyses were performed on a purified pool of adducts (see Experimental Section and Figure 2 for typical chromatograms). In short, we developed a quick purification step by using a Sep Pak® Vac cartridge; while this provided the pool of adducts in a non-saline water/acetonitrile solution, it did not allow us to separate each isomer (the different adducts have been separated by HPLC, but this method did not allow us to isolate sufficient amounts of each isomer to perform NMR analyses). After concentration to dryness under vacuum, NMR analyses in [D_6]DMSO by means of ^{1}H-^{1}H COSY, ^{1}H-^{13}C HMQC (^1J), and long-range (^3J) proton–carbon correlations (HMQC-LR) led to a nearly complete characterization of the molecules. The assignments of the chemical shifts of carbons (at least for the nicotinoyl and isonicotinoyl moieties) were made by performing two-dimensional ^{1}H-^{13}C HMQC experiments.

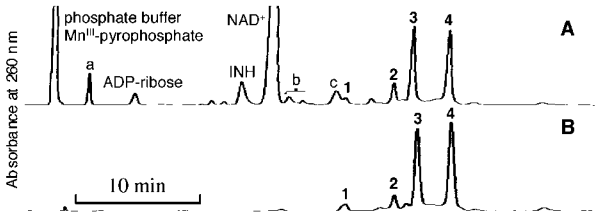

Figure 2. HPLC profiles of A) a crude mixture obtained by reacting INH (2 mM), NAD^+ (2 mM), and Mn^{III} pyrophosphate (4 mM) in phosphate buffer (100 mM, pH 7.5) for 20 min and B) a solution of the purified pool of adducts **1**–**4**. Signal assignment: a = isonicotinic acid, b = nicotinamide and isonicotinamide, c = traces of oxidized adduct.

NMR spectra of the purified pool of adducts show a mixture of four compounds with structures **1**–**4**. The main data supporting these structures are summarized below and reported in Figure 3 and Table 1. In the four derivatives **1**–**4**, the isonicotinoyl radical is bound at the 4-position of the nicotinamide ring, as deduced from ^{1}H-^{1}H COSY spectrum

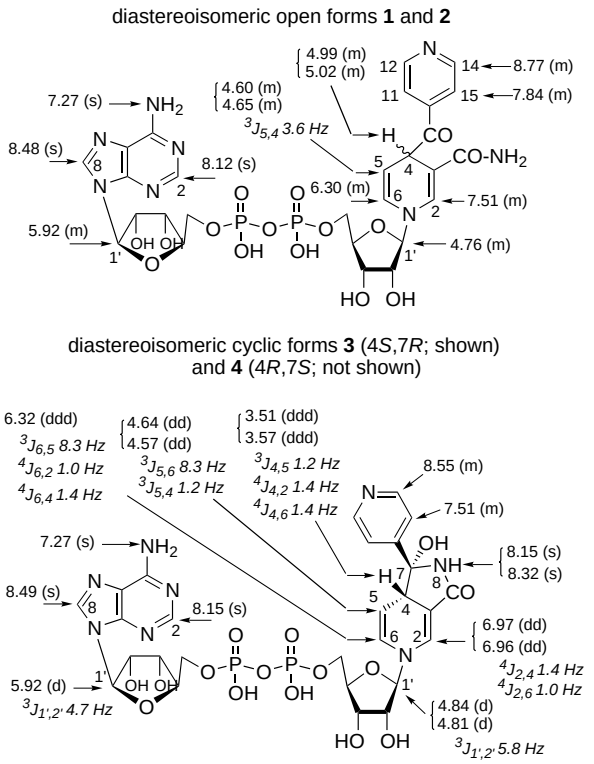

Figure 3. Detailed ^{1}H NMR data for INH–NAD(H) adducts **1**–**4** (when distinct signals for each diastereoisomer were detected, they are indicated with a bracket).

Table 1. Main ^{1}H and ^{13}C NMR signals for INH–NAD(H) adducts **1**–**4** in [D_6]DMSO solution. See Figure 1 for numbering of H and C atoms and Figure 3 for detailed ^{1}NMR data. (N)H1′ and(A)H1′ refer to H1′ from nicotinamide and adenine ribonucleotide moieties, respectively.

	Open		Cyclic	
	1	**2**	**3**	**4**
^{1}H NMR				
H2	7.51	7.51	6.97	6.96
H4	4.99 or 5.02	4.99 or 5.02	3.51	3.57
H5	4.60 or 4.65	4.60 or 4.65	4.64	4.57
H6	6.30	6.30	6.32	6.32
H7	–	–	?	?
H8	?	?	8.15	8.32
H11, H15	7.84	7.84	7.51	7.51
H12, H14	8.77	8.77	8.55	8.55
(N)H1′	4.76	4.76	4.84	4.81
(A)H1′	5.92	5.92	5.92	5.92
H2 (adenine)	8.12	8.12	8.15	8.15
H8 (adenine)	8.48	8.48	8.49	8.49
NH_2 (adenine)	7.27	7.27	7.27	7.27
^{13}C NMR				
C2	134.1 or 135.4	134.1 or 135.4	128.6	132.6
C3	105.1	105.1	102.7	104.0
C4	44.0	44.0	47.5	47.6
C5	100.0 or 100.5	100.0 or 100.5	98.9	97.5
C6	130.5 or 132.8	130.5 or 132.8	128.8	128.8
C7	199.2 or 199.6	199.2 or 199.6	88.5	88.8
C9	169.4	169.4	171.7	171.7
C10	165.2	165.2	153.9	153.9
C11, C15	122.7	122.7	121.5	121.5
C12, C14	151.5	151.5	150.2	150.2
(N)C1′	95.9	95.9	95.9	95.9
(A)C1′	87.7	87.7	87.7	87.7

and ^{1}J ^{1}H-^{13}C HMQC for the four H2, H4, H5, and H6 protons.

The following arguments support the open structures for **1** and **2** and cyclic ones for **3** and **4**:

1) H4 resonances are deshielded in the open structures **1** and **2** ($\delta = 4.99$ and 5.02 ppm, respectively) with respect to the cyclic ones **3** and **4** ($\delta = 3.51$ and 3.57 ppm, respectively) (Figure 3). These H4 resonances represent the more selective and convenient signal to distinguish the four isomers as shown in Figure 4. In pure [D$_6$]DMSO, the ratio of cyclic to open structures was 60:40, but it was increased up to 76:24 when spectra were recorded in D$_2$O (Table 2).

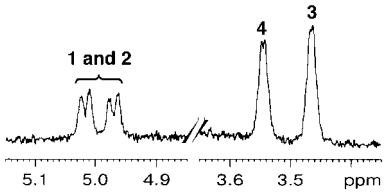

Figure 4. Parts of the 300 MHz ^{1}H NMR spectrum in [D$_6$]DMSO (plus one drop of D$_2$O) of the pool of adducts **1**–**4**, showing the characteristic signals of the four H4 key protons ($^{3}J_{4,5} = 3.6$ Hz for **1** and **2**, 1.2 Hz for **3** and **4**).

Table 2. Solvent effect on the ratio open:cyclic INH–NAD(H) adducts, determined from the ratio of NMR H4 signal areas (aqueous DMSO) or of H2 signal areas (D$_2$O) for **1** + **2** and **3** + **4** isomers.

	% of open isomers **1** + **2**	% of cyclic isomers **3** + **4**
[D$_6$]DMSO + 1 drop of D$_2$O	40	60
[D$_6$]DMSO:D$_2$O 75:25	30	70
[D$_6$]DMSO:D$_2$O 25:75	25	75
D$_2$O	24	76

2) The chemical shift of C7 is characteristic of a carbonyl group for **1** and **2** ($\delta = 199.2$ and 199.6 ppm, respectively) and of a tetrahedral carbon for **3** and **4** ($\delta = 88.5$ and 88.8 ppm, respectively)
3) As observed in the ^{1}H-^{13}C HMQC-LR spectrum of compounds **3** and **4** (Figure 5), strong ^{3}J correlation between H8 and C4 and weak but significant ^{2}J correlation between H8 and C7 support the cyclic structure; in the case of open structures, these H8-C4 and H8-C7 correlations should be assigned to highly unlikely ^{4}J and ^{5}J correlations, respectively.

It should be noted that neither the carboxamide protons H8 in **1** and **2** nor the hydroxylic H7 protons in **3** and **4** were detected, even in pure DMSO, probably because of the presence of some moisture (most of other signals of hydroxylic protons were also absent).

On the basis of one-dimensional ROE experiments (Figure 6), which show the spatial proximity of H4 with H11 and H15, both **3** and **4** have a *trans* configuration (the pyridine and dihydropyridine nuclei being on opposite sides of the imidazolidinone ring). Consequently, **5** and **6** (only detected by LC/MS) must have a *cis* configuration, and the low amount observed may be related to steric hindrance of the two bulky substituents.

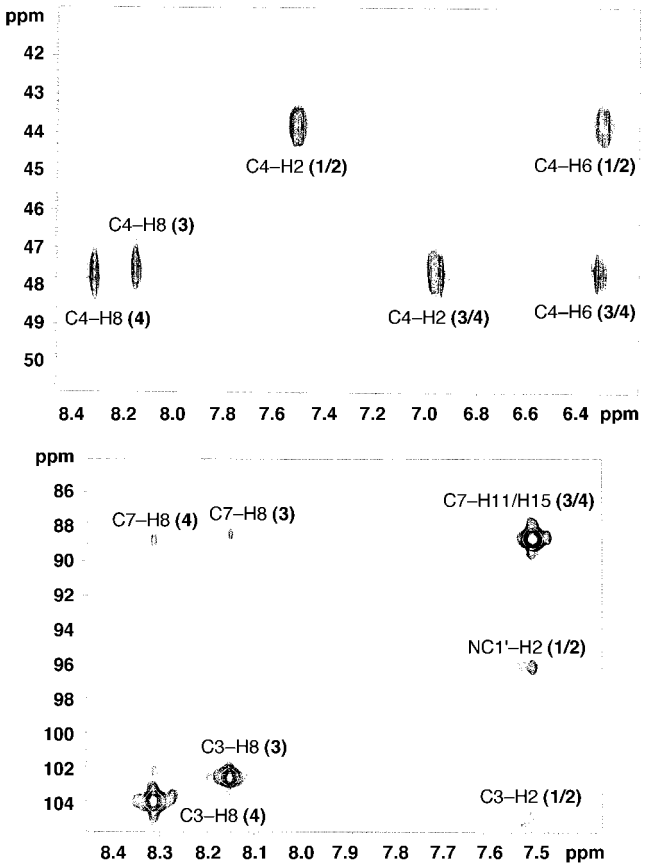

Figure 5. Two-dimensional ^{1}H-^{13}C HMQC long-range correlations for the mixture of compounds **1**–**4**.

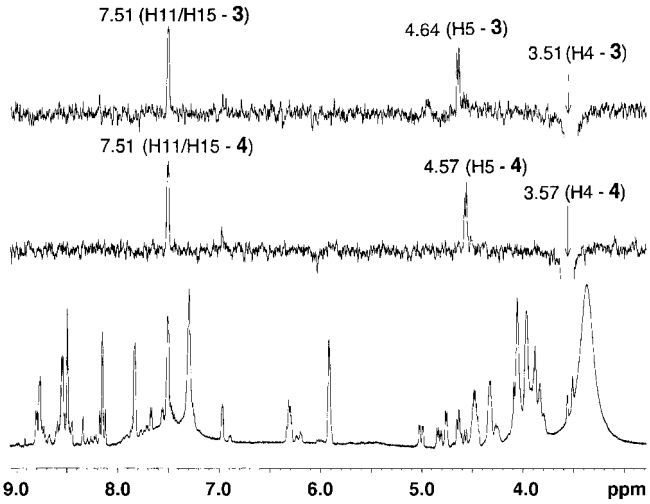

Figure 6. ROE difference spectra in compounds **3** and **4** between H4 and both H11, H15.

Furthermore, all the above NMR attributions are fully supported by analyses performed on simplified adducts obtained by reacting INH with NNA^{+}, a model of NAD^{+} in which the ADP ribose was replaced by the nonchiral substituent (2-acetoxyethoxy)methyl, therefore reducing the number of adducts. Only the open adduct **7** (in fact the

racemic mixture 4*R* and 4*S*) and the cyclic adduct **8** (in fact the racemate with *trans* configuration, i.e., the pair of enantiomers 4*S*,7*R* and 4*R*,7*S*) were observed (Figures 7 and 8 and Table 3). Compound **9** was present in too low an amount to be detected by NMR spectroscopy.

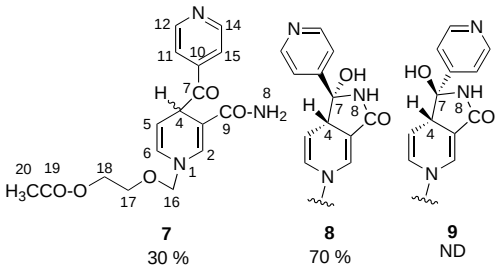

Figure 7. Structures of the covalent adducts isoniazid–NNA(H). Compound **7** is the pair of enantiomers 4*R* and 4*S*; compound **8** is the pair of enantiomers 4*S*,7*R* (shown) and 4*R*,7*S* (not shown); compound **9** is the pair of enantiomers 4*S*,7*S* (shown) and 4*R*,7*R* (not shown). The yields are estimated yield from NMR analysis; ND = not detected by NMR spectroscopy.

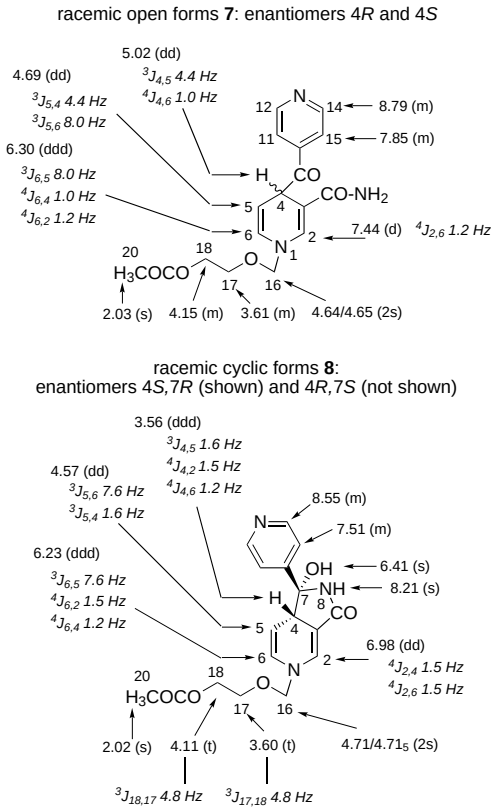

Figure 8. Detailed ^{1}H NMR data for INH–NNA(H) adducts **7** and **8**.

Oxidized adducts, which are only formed as traces in the present conditions (peak c in Figure 2), have been previously observed,[4] and the question of their exact structure has been raised. Preliminary NMR results (not shown) performed on a concentrated sample of these oxidized adducts showed the coexistence of two diastereoisomeric cyclic adducts.

So, as previously proposed on the basis of LC-MS analyses and isotopic experiments, the present ^{1}H and ^{13}C NMR analyses demonstrate that INH–NAD(H) adducts coexist in solution as both open and cyclic structures. Open adducts **1** and **2** consist of addition of an isonicotinoyl radical at the 4-position of the nicotinamide moiety of the coenzyme (creating a new chiral center at the C4 position that gives two diastereoisomers). From these adducts, the cyclization resulting of intramolecular addition of the amino group of nicotinamide on the carbonyl of the isonicotinoyl residue affords hemiamidal structures; creation of the new chiral C7 carbon (in addition to C4) is consistent with the four observed diastereoisomers, but only two of them were in sufficient amounts to be analyzed by NMR spectroscopy.

Table 3. Main ^{1}H and ^{13}C NMR signals (δ [ppm]) for the two INH–NNA(H) adducts **7** and **8**, one open and one cyclic, detected in solution (solvent [D$_6$]DMSO). For numbering of H and C atoms, see Figure 7.

	Open **7**	Cyclic **8**
^{1}H NMR		
H2	7.44	6.98
H4	5.02	3.56
H5	4.69	4.58
H6	6.30	6.23
H7	–	6.41
H8	?	8.21
H11, H15	7.85	7.51
H12, H14	8.79	8.55
H16	4.64, 4.65	4.71, 4.71(5)
H17	3.61	3.60
H18	4.15	4.11
H20	2.03	2.02
^{13}C NMR		
C2	137.5	132.8
C3	104.3	103.4
C4	43.0	47.1
C5	101.0	98.1
C6	131.0	132.3
C7	198.9	88.9
C9	169.3	171.6
C10	?	153.8
C11, C15	122.7	121.6
C12, C14	151.5	150.3
C16	83.0	83.2
C17	66.1	65.9
C18	63.8	63.9
C19	171.2	171.2
C20	21.6	21.6

Since a mixture of these different adducts was shown to give efficient competitive inhibition of InhA with partial results indicating different level of activity for each of the two main adducts **3** and **4**,[9] and although the structure of the bound inhibitor proposed by Rozwarski et al. on the basis of a X-ray crystallography study with a resolution of 2.7 Å was an open structure,[3] it is legitimate to raise the question about the effective active form(s) of INH–NAD(H) adduct(s): are there several forms able to inhibit the InhA activity or is there a single active form mainly endowed with inhibitory potency? Could cyclic hemiamidal diastereoisomers, which are the main products present in aqueous solution, behave as real InhA inhibitors? These studies will also be useful to help further design of specific inhibitors for the development of new antituberculosis agents.

Experimental Section

Synthesis of *N*-[(2-acetoxyethoxy)methyl]nicotinamide bromide (NNA^+): Treatment of 1,3-dioxolane (100 mmol) with acetyl bromide (106 mmol) gave (2-acetoxyethoxy)methyl bromide in 82% yield.[10] (2-Acetoxyethoxy)methyl bromide (250 mg, 1.27 mmol) was added to a stirred solution of nicotinamide (122 mg, 1 mmol) in dry THF (6 mL) under argon and at RT. After 90 min, the white precipitate was isolated, washed three times with THF, and dessicated under vacuum to give 277 mg of *N*-[(2-acetoxyethoxy)methyl]nicotinamide bromide (NNA^+, 88% yield). ^{1}H NMR (300 MHz, [D_6]DMSO, 25 °C, TMS): $\delta = 1.99$ (s, 3H; $COCH_3$), 3.89 (t, 2H; OCH_2CH_2), 4.15 (t, 2H; OCH_2CH_2), 8.22 (s, 1H; NH), 8.37 (dd, $^3J = 8$, 6 Hz, 1H; H5), 8.67 (d, $^3J = 8$ Hz, 1H; H6), 9.27 (d, $^3J = 6$ Hz, 1H; H4), 9.55 (s, 1H; H2). MS (ESI): m/z: 239.0 [M^+].

Preparation of the INH–NAD(H) and INH–NNA(H) adducts and purification of the pool of adducts. The reaction medium (15 mL final volume) contained INH (2 mM), NAD^+ (2 mM) or NNA^+ (2 mM) and Mn^{III} pyrophosphate (4 mM; prepared as described in ref. [8]) in phosphate buffer (100 mM, pH 7.5) and was stirred at RT for 20 min. The pool of adducts [**1**–**6** for INH–NAD(H)] or **7**–**9** for INH–NNA(H) was separated from other components of the reaction mixture (INH, NAD^+ or NNA^+, ADP-ribose, isonicotinic acid, isonicotinamide, nicotinamide, and buffer) by using a Sep Pak® Vac 20 cc (5 g) cartridge. The reaction mixture (15 mL) was loaded in the cartridge and washed with 4 mM NH_4OAc aqueous solution; only the pool of adducts was retained. Careful washing with water followed by elution with acetonitrile and concentration to dryness under vacuum afforded the pool of adducts. After dilution in [D_6]DMSO for NMR analysis, a control HPLC injection of this pool of adducts displayed a profile similar to the adduct profile observed in the crude reaction mixture (see typical chromatograms of INH–NAD(H) adducts in Figure 2). The oxidized adduct (conversion of dihydropyridine to the pyridinium structure) previously described during KatG oxidation of INH in the presence of NADH[4] was only detected as traces (less than 5%) in HPLC analyses and was completely eliminated during the purification process. HPLC analyses were performed on a reverse-phase C18 column (nucleosil, 10 μm, 250 × 4.6 mm) by using a linear gradient from 100% NH_4OAc (70 mM) aqueous solution to 20% acetonitrile for INH–NAD(H) adducts analyses and to 100% methanol for INH–NNA(H) adducts analyses (flow rate: 1 mL min^{-1}). The column was coupled to a diode array detector (Kontron) for the detection of products at 260 nm and the monitoring of UV-visible spectra.

Acknowledgements

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