# THE PRIMARY ACID PRODUCT OF DPNH

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SUMMARY - Analysis of the proton magnetic resonance spectra obtained at 220 MHz confirms the axial conformation of the C-6 hydroxyl in the model primary acid product 1-n-(2,6-dichlorobenzyl)-6-hydroxy-1,4,5,6-tetrahydronicotinamide. In the primary acid product of DPNH however the reaction occurs stereospecifically with the substitution at the C-6 position equatorial and on the B-side of the pyridine ring and the C-4A proton axial. A cyclic structure $\alpha,O^{2'}$-6B cyclo-tetrahydronicotinamide is proposed for the primary acid product of DPNH, formed by epimerization of βDPNH to the α configuration followed by protonation at C-5 and subsequent attack of the ribose C-2'-OH on the C-6 position forming a new five membered ring.

Speculation has arisen involving a possible relationship between oxidative phosphorylation and 6-hydroxy-tetrahydronicotinamide (6-HTN) systems (1,2). While the structure of the model 6-HTN has been convincingly demonstrated (3-5), it has been assumed that the primary acid product of DPNH also has an analogous structure (2,6,7). Circular dichroism studies have reported that αDPNH and βDPNH both yield the identical primary acid product and that this product has an α configuration about the pyridine-ribose linkage (7). These findings would seem to indicate that more is involved in the reaction than just a hydration of the 5-6 double bond of the dihydronicotinamide ring of DPNH.

METHODS - Dichlorobenzyl-nicotinamide,-dihydronicotinamide, and 6-HTN were synthesized as described by Kim and Chaykin (4) and $DPNH_BD_A$ and $DPNH_AD_B$ were made as described by Oppenheimer *et al.* (8). The primary acid product of DPNH was formed by lowering the pH of an unbuffered 10 mM DPNH solution to pH 3.0

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Abbreviations: DPNH', the primary acid product of DPNH; $DPNH_BD_A'$, $DPNH_AD_B'$ the primary acid product of the specific deuterium label on the A and B side respectively of DPNH. 6-HTN, dichlorobenzyl 6-hydroxy-tetrahydronicotinamide; TMS, tetramethylsilane; TSP, trimethylsilyl tetradeutero sodium propionate.

with 1 N HCl and incubating the solution until the $OD_{340}$ declined to less than 5% of the original value. The reaction was then quenched with ammonium bicarbonate, lyophylized and eluted from a DEAE column with a 5 mM-500 mM ammonium bicarbonate gradient.

The NMR spectra were recorded on a Varian HR 220 Spectrometer, signal to noise enhanced with a Nicolet 1074 computer and spin decoupled with a Wavetek 131A Voltage-Controlled Generator. Samples were routinely run at a 50 mM concentration, pD 8.1, and at 22° C.

RESULTS - 1-n-(2,6-dichlorobenzyl)-6-hydroxy-1,4,5,6-tetrahydronicotinamide - The assignments of the two methylene groups were made by conducting the primary acid reaction in $D_2O$ which assigns the C-5 protons, and by using the Karplus relation for adjacent methylene protons in a six membered ring where a trans conformation (axial-axial) has a 10-13 Hz coupling constant and a gauche conformation (axial-equatorial or equatorial-equatorial) has a 2-6 Hz coupling constant. The spectra of the C-4 and C-5 protons of 6-HTN (Fig. 1) consists of a triplet, two doublets and a triplet, all extensively split by many smaller couplings among the protons of the ring. Specific coupling constants (Table 1) were confirmed by spin decoupling experiments. The large coupling constant of 13.0 Hz between the C-4 proton at 494 Hz and the C-5 proton at 245 Hz indicate that these protons are trans to each other and therefore must both be axial. The remaining protons at 440 Hz and 350 Hz are the C-4 and C-5 equatorial protons respectively; their small coupling constants listed in Table 1 are indicative of an equatorial-axial and an equatorial-equatorial spin-spin coupling and are completely consistent with these assignments. The chemical shifts of these protons are given in Table 2.

The small and equal coupling of 2.5 Hz between the C-6 proton at 1142 Hz and the C-5 axial and C-5 equatorial protons indicate that the C-6 proton is equatorial and that the hydroxyl group must therefore be axial. Thus, the results of the detailed spectral analysis of the coupling constants confirm in solution the structure determined from the crystalline solid using x-ray diffraction (3).

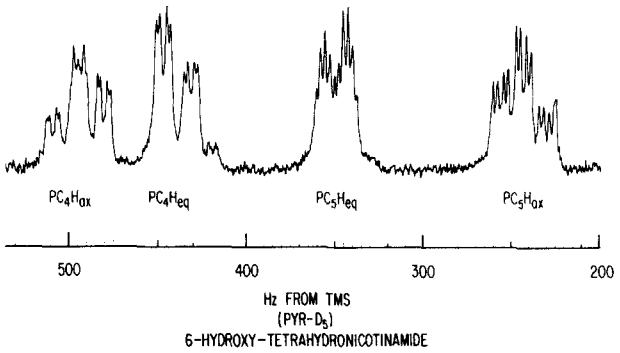


Figure 1: The C-4 and C-5 methylene protons of Dichlorobenzyl 6-Hydroxy-tetrahydronicotinamide, 22° C in Pyr $D_5$ at 220 MHz.

TABLE 1

COUPLING CONSTANT

| Nuclei Coupled | 6-HTN[a] | $DPNH_AD'_B$ [b] | $DPNH_BD'_A$ [b] |
|---|---|---|---|
| $J_{6-5ax}$ | 2.5 | 9.5 | 9.5 |
| $J_{6-5eq}$ | 2.5 | 4.2 | 4.2 |
| $J_{5ax-4ax}$ | 13.3 | 13.0 | -- |
| $J_{5ax-4eq}$ | 6.0 | -- | 5.3 |
| $J_{5ax-5eq}$ | 13.1 | 11.7 | 11.7 |
| $J_{5eq-4ax}$ | 5.6 | 5.4 | -- |
| $J_{5eq-4eq}$ | 2.0 | -- | 2.0 |
| $J_{4ax-4eq}$ | 15.8 | -- | -- |
| $J_{4ax-2}$ | 2.0 | 1.8 | -- |

Coupling Constants ± 0.2 Hz

[a] in $PyrD_5$

[b] in $D_2O$

It should be noted that the model compound is a mixture of two isomeric forms generated by the asymmetric center at the C-6 position. Because these

TABLE 2

CHEMICAL SHIFT

| | 6-HTN[a] | DPNH'[b] |
|---|---|---|
| $PC_2H$ | 1732 | 1592 |
| $PC_6H$ | 1142 | 1098 |
| $PC_{5ax}$ | 245 | 278 |
| $PC_{5eq}$ | 350 | 485 |
| $PC_{4ax}$ | 494 | 450 |
| $PC_{4eq}$ | 440 | 492 |

Chemical shift values ± 1 Hz obtained at 22° C and for 50 mM solutions, at 220 MHz.

[a] in Pyr $D_5$, Hz from TMS

[b] in $D_2O$, Hz from TSP

two forms are enantiomeric, i.e., mirror images, they are magnetically equivalent and therefore indistinguishable by NMR (9).

DPNH'. The chemical shifts for the primary acid product of DPNH are markedly different; as can be seen from Table 2. The pyridine C-4 protons were assigned by specifically labeling the DPNH enzymatically with deuterium. The pyridine C-5 protons were assigned by chemical shift analogy with the 6-HTN model compound, performing the reaction in $D_2O$, and by the coupling constants to the C-4 protons. The coupling constants of the C-6 proton, $J_{6-5ax}$ of 9.5 Hz and $J_{6-5eq}$ of 4.2 Hz are characteristic of an axial-axial and an axial-equatorial conformation respectively.

Two important features are immediately apparent: 1.) The coupling constants and hence the geometric environment of the pyridine C-6 proton are quite different between 6-HTN and DPNH' (Fig. 2). In the model compound the pyridine C-6 proton is equatorial; gauche to both of the C-5 protons and the OH is axial. In DPNH', the C-6 proton is axial implying an equatorial substitut-

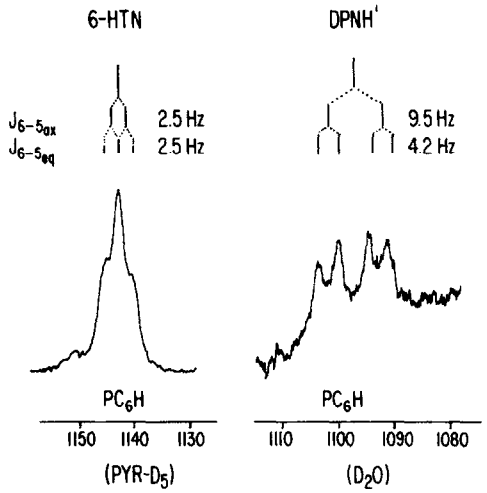


Figure 2: Comparison of the splitting pattern for the PC-6 proton of 6-HTN and DPNH'. The two small and equal coupling constants to the C-5 protons in 6-HTN indicate a gauche-gauche conformation while the large and small coupling constants to the C-5 protons of DPNH' indicate a trans-gauche conformation. The spectra were recorded at 220 MHz.

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ion at the C-6 position. 2.) DPNH' formation is stereospecific. The two possible diastereomers formed by attack from either side at the C-6 position could be distinguished by the spin-spin coupling constant of the $C\text{-}5_{ax}$ proton to the enzymatically prepared specifically labelled C-4 proton even if the chemical shifts of all the protons involved were fortuitously identical. This is because attack at the C-6B position in $DPNH_AD_B$ for example would force the C-4A proton axial therefore giving a large trans coupling to the $C\text{-}5_{ax}$ proton while attack on the opposite side would force the C-4A proton equatorial hence giving a small gauche coupling constant to the $C\text{-}5_{ax}$ proton. If the two possible diastereomers were present these different coupling constants could be measured in principle because their respective spectra would be superimposed. In fact only one form and one set of coupling constants are observed, with the C-4A proton trans to the $C\text{-}5_{ax}$ proton (Fig. 3) thus indicating that the reaction is stereospecific.

DISCUSSION. It is quite evident that the structure of DPNH' is not the same

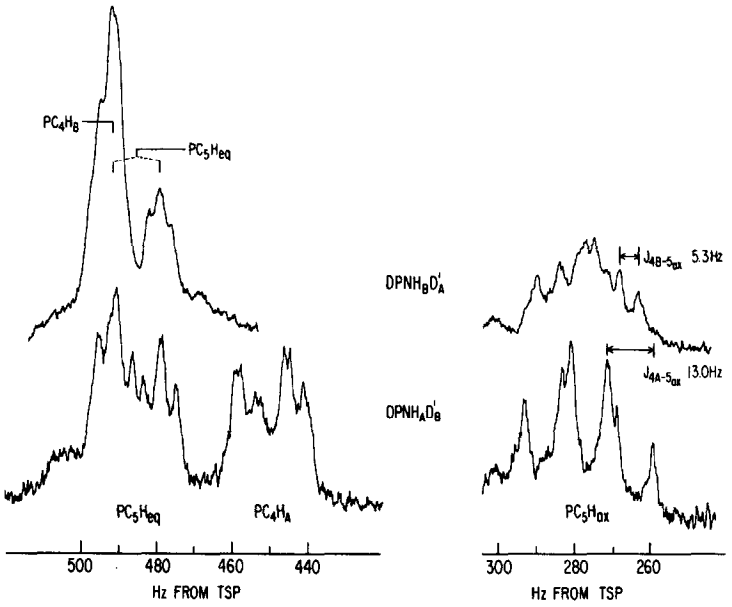


Figure 3: The tetrahydronicotinamide protons of the specifically labelled $DPNH_BD'_A$ (top) and $DPNH_AD'_B$ (bottom) in $D_2O$, 22° C, at 220 MHz.

as that of the model system. The proposed structure, Figure 4, is an attempt to explain the fact that the reaction occurs stereospecifically and that the C-6 substitution is equatorial. While an exact sequence of the reaction is currently under investigation, the overall reaction involves an acid catalyzed epimerization and then protonation at the C-5 position followed by attack of the C-2' ribose hydroxyl on the C-6 position forming a five membered ring.

This structure is attractive in that it explains many of the results that have been reported for DPNH'. The epimerization to the α form and the closing of the five membered ring explains why αDPNH and βDPNH both yield the identical compound (7) and why that compound should have an α configuration.

The stereospecificity for this reaction is built into the molecule. The structure as described with the $O^{2'}$-C-6 substitution equatorial and on the B-side of the pyridine ring would be obtained by the attack of the C-2' hydroxyl on the anti,α-tetrahydronicotinamide cation intermediate while its plane is at right angles to the plane of the ribose ring, a minimum energy conformation

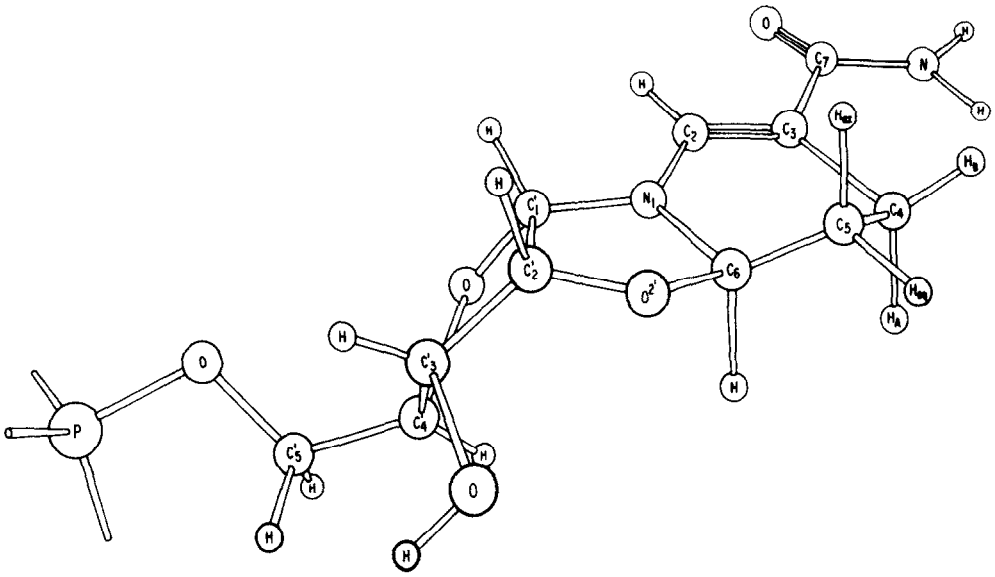

Figure 4: The proposed absolute configuration of the tetrahydronicotinamide ring in DPNH'. The C-5ax proton is trans to the C-6 proton and C-4A proton and gauche to the C-4B proton. The $O^{2'}$ of the ribose is equatorial and on the B-side of the tetrahydronicotinamide ring.

about the glycosidic bond. Attack by the C-2'-OH on the A-side of the pyridine ring would require the pyridine ring to be coplanar with the ribose ring, an energy maximum (10). An energy difference of as little as 1.3 kcal between these two intermediate forms would essentially make the amount of the energetically unfavorable form so small (<10%) as to be not easily detected in the presence of the major form by PMR.

More detailed studies are presently underway to investigate the stereospecificity of this reaction and to relate the structure of the primary acid product of DPNH to the enzyme product of triose phosphate dehydrogenase, DPNHX. These results point out the great structural and hence chemical differences between 6-HTN and DPNH' and raise questions about the simple extrapolation of the reactions and properties of model dihydronicotinamide compounds to DPNH.

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