Communication

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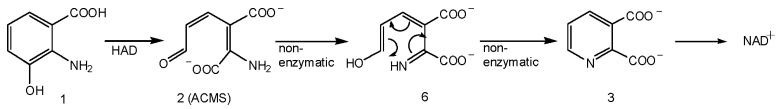

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The Pyridine Ring of NAD Is Formed by a Nonenzymatic Pericyclic Reaction

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The biosynthesis of quinolinate **3**, the precursor to the pyridine ring of NAD, is still poorly understood. Two pathways have been identified, one involving the direct formation of quinolinic acid from aspartate and dihydroxyacetone phosphate,[1] the other requiring a five-step degradation of tryptophan.[2] The final step in this degradation is catalyzed by the non-heme Fe(II)-dependent enzyme 3-hydroxyanthranilate-3,4-dioxygenase (HAD). This enzyme catalyzes the oxidative ring opening of 3-hydroxyanthranilate (**1**) to 2-amino-3-carboxymuconic semialdehyde (ACMS, **2**) which then cyclizes to quinolinate (**3**). Until recently, quinolinate formation from **1** was difficult to study because HAD was an unstable, low-abundance protein in all sources examined.[3−6] However, recent identification and characterization of the stable *Ralstonia metallidurans* HAD, which can be overexpressed at a high level in *Escherichia coli*, has greatly facilitated the mechanistic characterization of this system.[2] In this communication we describe the solution structure of the most stable isomer of **2** and the mechanisms for its isomerization and conversion to **3**.

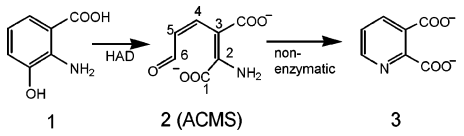
Previous studies, using low concentrations of partially purified beef kidney HAD to generate ACMS, suggested that the cyclization of ACMS (**2**) to quinolinate (**3**) is not enzyme catalyzed.[7] We have confirmed this by treating HAD-free ACMS, readily prepared by the enzymatic oxidation of **1** followed by chloroform precipitation and ultrafiltration, with high concentrations of active, purified HAD. The "enzymatic" and nonenzymatic cyclization rates were 0.011 min^{-1} and 0.015 min^{-1}, respectively. While this experiment clearly demonstrates that the cyclization of ACMS is not catalyzed by HAD, it does not rule out the possibility that a separate, as yet uncharacterized protein, catalyzes this reaction.

The conversion of **2** to quinolinate involves isomerization to **4** (see below) followed by cyclization to **7** and dehydration to quinolinate **3** (Scheme 2). Two mechanisms have been proposed for the cyclization reaction.[7] In mechanism A, the amine undergoes a nucleophilic addition to the aldehyde to give **5**. This mechanism poses several problems: (1) the amine lone pair is poorly oriented for overlap with the antibonding orbital of the aldehyde, (2) this amine is a weak nucleophile having the reactivity of a vinylogous amide rather than that of an amine, and (3) electron donation from the amine to the aldehyde carbon attenuates the electrophilicity of this carbon, making it more like an amide than an aldehyde carbonyl. This analysis is supported by the low reactivity of ACMS (**2**) with strong nucleophiles such as hydroxylamine and by the low basicity of the amine ($pK_a < 5$).[7] On the basis of these considerations, we expect that cyclization of **4** by the nucleophilic addition mechanism is likely to be very slow. An alternative that overcomes these problems is shown in mechanism B in which **4** tautomerizes to **6**, which can then undergo a 6π electrocyclization reaction to give **7**. Previous efforts to differentiate between these mechanisms using isotope effects, pH-rate studies and acid−base catalysis were unsuccessful.[7]

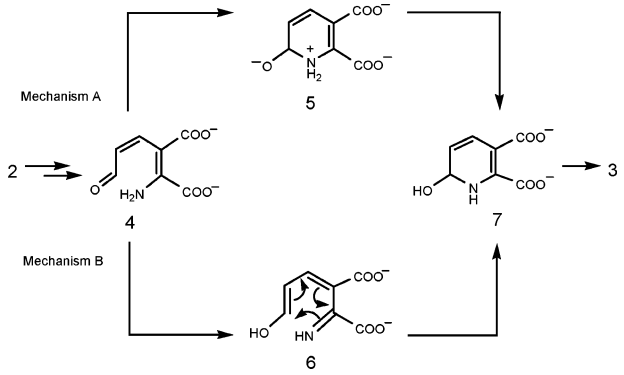
N,*N*-Dimethylcarbamoylpyridinium (**8**) undergoes facile ring opening to give a product assumed to be **12**.[8,9] This reaction involves the addition of hydroxide to the pyridinium ring followed by ring opening. This ring opening is a model for the microscopic reverse of the conversion of **4** to **7**. It should be possible to differentiate between ring opening by an elimination (**9** to **10** in Scheme 3—the reverse of **4** to **5** in Scheme 2) and ring opening by a pericyclic reaction (**9** to **11** via mechanism B in Scheme 3—the reverse of **6** to **7** in Scheme 2) by comparing the rates of the hydroxide- and methoxide-mediated reactions. For hydroxide, facile ring opening could occur by both mechanisms, while for methoxide, ring opening by mechanism A is expected to be much slower than ring opening by mechanism B because methoxy is a much weaker electron donor than alkoxy.

When *N*,*N*-dimethylcarbamoylpyridinium **8** was treated with hydroxide (0.7 equiv) in D_2O, rapid ring opening occurred. NMR analysis of the reaction mixture demonstrated the formation of two products **14** and **15**, which were easily differentiated based on the chemical shifts of the aldehydic and the corresponding enol protons

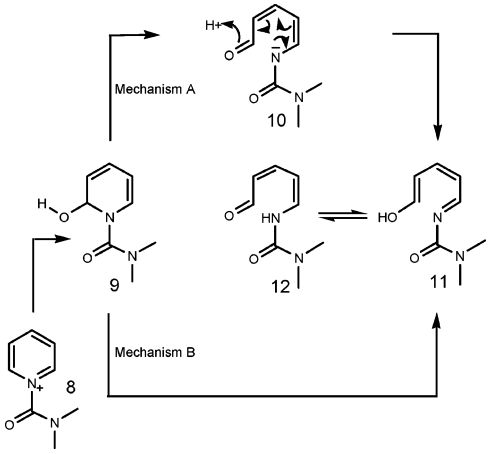
Scheme 1



Scheme 2



Scheme 3



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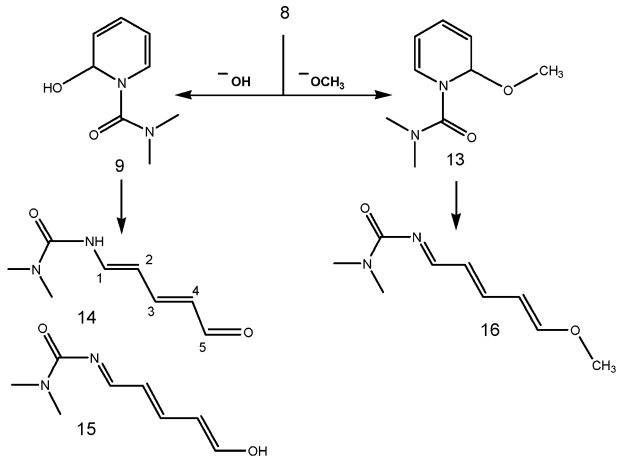
Scheme 4



at 9.04 and 7.54 ppm, respectively (Scheme 4). Treatment of *N*,*N*-dimethylcarbamoylpyridinium **8** with methoxide (0.7 equiv) in CD_3OD also resulted in rapid ring opening. NMR analysis of this reaction mixture demonstrated the formation of **16** as the major product (Scheme 4). The chemical shift of the C5 vinyl proton of **16** in D_2O (7.53 ppm) was consistent with the corresponding assignment of the C5 proton of **15** (7.54 ppm). In both cases complete reaction occurred within the mixing time, and no rate difference between the two reactions was evident. This suggests that the ring opening of **9** is occurring by a pericyclic reaction and supports the hypothesis that the conversion of **4** to **7** is also occurring by a pericyclic reaction.

The coupling constants for H1−H5 for compounds **14, 15**, and **16** are consistent with the trans stereochemistry for each of the CC double bonds, suggesting that the initially formed cis isomer **12** undergoes facile rotation about the C1−C2 and the C3−C4 bonds. To determine the solution structure of ACMS (**2**), it was generated by the HAD-catalyzed oxidation of **1** in D_2O buffer, and the reaction mixture was analyzed by NMR (Figure 1). This analysis demonstrated that ACMS was formed as a single isomer (Figure 1). This isomer is likely to be the aldehyde rather than the enol tautomer because the chemical shift of the C6 proton (8.8 ppm) is closer to that of the C5 proton of **14** (9.04 ppm) than the C5 proton of **15** (7.54 ppm) or **16** (7.53 ppm). The coupling constants between the H4, H5, and H6 protons of ACMS are consistent with a trans C4−C5 double bond. This was confirmed by the observation of an NOE between H4 and H6. The ^{1}H NMR analysis does not enable us to assign the stereochemistry about the C2−C3 double bond of ACMS. However, since both carboxylates are ionized at pH = 7 and ACMS is conformationally mobile, it is likely that these groups are trans to minimize electrostatic repulsion. We therefore suggest that the all trans isomer of ACMS (**17**) represents the most stable stereoisomer in solution.

The all trans stereoisomer of ACMS (**17**) must undergo two cis/trans isomerization reactions, one about the C2−C3 double bond, the other about the C4−C5 double bond, before it can undergo the cyclization reaction to give **7**. These isomerization reactions do not involve ACMS protonation at C5 because ACMS does not undergo H/D exchange with buffer and no solvent deuterium is incorporated into the quinolinic acid. (Figure 1). We therefore suggest that ACMS (**17**) is in equilibrium with a small concentration of its enol tautomer **18** and that the required double bond rotations occur from this tautomer (Scheme 5). This is consistent with the observations that **12** rapidly isomerizes to **14** and that **14** is in equilibrium with **15** (Schemes 3 and 4).

Our studies confirm that the conversion of ACMS to quinolinate is not enzyme catalyzed. In addition, we propose that the all trans

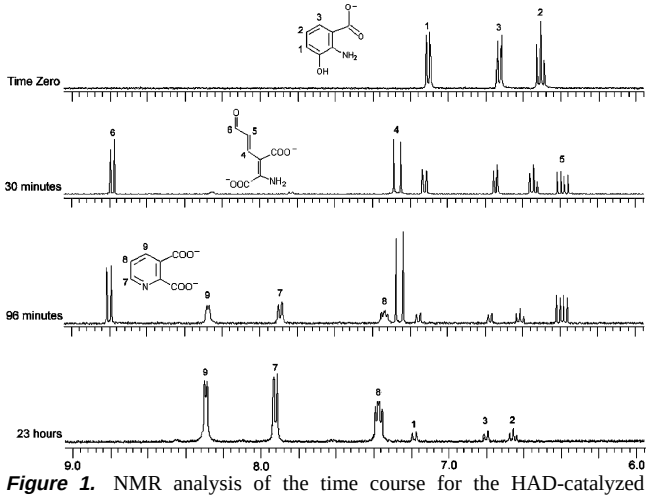
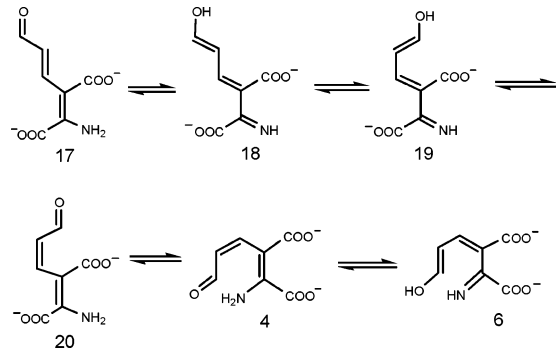


Figure 1. NMR analysis of the time course for the HAD-catalyzed conversion of 3-hydroxyanthranilate (**1**) to quinolinate (**3**). After 30 min peaks due to a single isomer of ACMS were evident; after 96 min, most of the substrate was consumed, and peaks due to both ACMS and quinolinate were evident. After 23 h, all of the substrate was converted to quinolinate.

Scheme 5



isomer **17** is the most stable form of ACMS in solution and that **17** undergoes facile cis to trans isomerization about the C2−C3 and C4−C5 double bonds via transient formation of its enol tautomer **18**. A model study on the ring opening of dimethylcarbamoylpyridinium with hydroxide and methoxide suggests that the cyclization of ACMS occurs by an electrocyclization reaction of the enol tautomer **6**. Thus, the biosynthesis of quinolinic acid by the tryptophan pathway is likely to be a member of a growing family of natural products whose biosynthesis involves a pericyclic reaction.[10]

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Supporting Information Available: NMR spectra and experimental preparations of HAD, ACMS, **8**, and **14**−**16**. This material is available free of charge via the Internet at http://www.pubs.acs.org.

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