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Laboratories and Demonstrations

The Synthesis, Identification, and Kinetic Characterization of the Photoaffinity Label: 3-Azidopyridine Adenosine Dinucleotide: An Advanced Undergraduate Laboratory Experiment Appropriate for Organic, Biochemistry, or Bio-organic Chemistry.

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Pioneering work by Westheimer over 30 years ago demonstrated the capability of the technique by labeling the active site of an enzyme.

We present here a complete photochemical experiment suitable for biochemistry, bio-organic, and organic chemistry laboratories. It provides experiences in chemical and enzymatic syntheses, spectroscopy (IR, NMR, UV), chromatography (TLC, GC-MS), and a simple enzyme kinetic study utilizing UV spectroscopy. The application of light energy to produce chemical changes has recently expanded beyond photography, lithography, and organic synthesis to include use in tumor phototherapy [1] and as antiviral agents [2]. Dyes and a variety of photoactive chemicals have demonstrated potential use as environmentally benign insecticides, herbicides, and fungicides [3, 4]. Nevertheless, the traditional undergraduate chemical laboratory curriculum provides little exposure to photochemistry.

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Background

Photoaffinity labeling (PAL) is the use of light to electronically excite chemicals which subsequently bond to target molecules. More precisely, appropriately designed and engineered photoactive reagents can label specific sites on molecules. Pioneering work by Westheimer over 30 years ago demonstrated the capability of the technique by labeling the active site of an enzyme [5]. Since those early experiments, expansion of photochemical techniques has fostered the increased utilization of PAL techniques in probing biological targets and systems. The structure and functions of lipids, steroids, proteins, RNA, DNA, and cellular organelles, in addition to a host of enzymes, have been investigated using these techniques.

Identification of the active site is crucial to the elucidation of the chemistry and mechanism of an enzyme and equally critical for attempts to control or regulate its function(s). A logical PAL candidate for an enzyme, possessing the requisite affinity, would be a naturally occurring prosthetic group of the enzyme, such as a coenzyme. Of the more than 2100 known enzymes, over half require a coenzyme such as nicotinamide adenosine dinucleotide (NAD^+). Since Westheimer's initial studies, NAD^+ has been variously modified with photolabile adducts and continues to afford great utility in enzyme characterization studies [6, 7].

3-Azidopyridine adenosine dinucleotide was one of the first NAD^+ analogs synthesized as a photoaffinity label for identifying the active site of NAD^+-dependent enzymes, such as yeast alcohol dehydrogenase (YADH). Since NAD^+ participates in the enzymatic reaction at the active site, a photoactive analog should possess a high degree of affinity. Upon irradiation of the 3-azido NAD^+ analog with UV light, a very reactive nitrene intermediate is formed, which can insert itself into a variety of chemical bonds including the C–H bond. Such insertion leads to the formation of a stable bond. A suitably tagged (i.e., fluorescent marker) 3-azido NAD^+ analog, bonded to the enzyme, thus becomes a photoaffinity label.

The synthesis and characterization of this photoaffinity label integrates photochemistry, synthetic chemistry, spectroscopy, and chromatography into a multiphase project. This project can be modified to meet constraints of time, instrumentation, student interest, and instructor's background. Further, this

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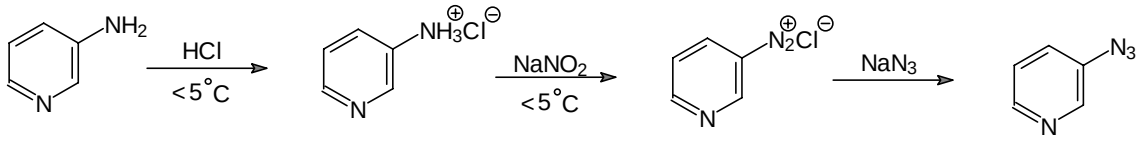

FIGURE 1. SYNTHESIS OF 3-AZIDOPYRIDINE [9].

multiphase project is patterned after work done by Hixson and Hixson [8], but significant alterations in protocol, instrumentation, and reagent concentrations have been made to meet the intended educational goals.

Experimental

Synthesis of 3-Azidopyridine (3 hr.)

3-Azidopyridine is prepared by diazotization of 3-aminopyridine [9] as shown in Figure 1.

3-Aminopyridine (4.70 g, 50 mmol) is stirred into concentrated HCl (12 mL), and the solution is chilled to 0 °C in ice water (brine). To the stirred slurry of the hydrochloride salt, $NaNO_2$ (3.8 g, 55 mmol) in ice water (22 mL) is added dropwise over a 20-min period, dissolving the hydrochloride salt. The clear solution is poured into a stirred slurry of NaN_3 (4.9 g, 75 mmol) and NaOAc (10.8 g) in ice water (10 mL). Vigorous nitrogen evolution should occur, and a red oil separates. After 10 min, the mixture is basified (NH_3) and then extracted with chloroform or pentane (100 mL then 3 × 25 mL). The combined chloroform (pentane) extracts are washed with 5% NaOH (2 × 25 mL) and water (25 mL) and then dried (Na_2SO_4). Removal of the solvent with a rotary evaporator affords a golden oil. Typical yield is 3.1 g (51%). It is suggested that IR, NMR, and GC-MS characterization of the 3-azidopyridine be done during the next laboratory period. (See Separation and Characterization section below.) IR ($CHCl_3$) $\tilde{\nu}_{max}$ (cm^{-1}) 2135, 2099 (N_3). GC-MS (optional) [HP5890, 30 m × 0.25 mm SPB-20 (film thickness 0.26 μm) fused silica capillary, GC column retention time: 5.88 min]: 120 (13.1, M^+); 92 (100, M–N_2); 65 (72.0). ^{1}H NMR (250 MHz, $CDCl_3$, δ): 7.3 (m, 3H), 8.4 (m, 1H). ^{13}C NMR (63MHz, $CDCl_3$, δ): 146.1 (C_2), 141.4 (C_6), 137.2 (C_3), 126 (C_5), 124.3 (C_4).

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Azides are sometimes explosive when neat and/or heated. Although azidopyridines are generally more stable than phenylazides, cautions must be taken when synthesizing the azido compound. A sufficiently pure compound can be obtained by simply removing the solvent, it is recommended *not* to further purify the compound by distillation. A shield must be used when rotary evaporating the solvent.

Synthesis of the 3-Azido NAD^+ Analog

The enzymatic synthesis of 3-azido NAD^+ involves an exchange reaction between the nicotinamide group of NAD^+ and 3-azidopyridine [8] catalyzed by porcine brain NADase (NAD^+ glycohydrolase, Sigma) as depicted in Figure 2. An incubation mixture is prepared by combining in a 5:1 ratio, 3-azidopyridine (0.066 g, 100 μmol) and NAD^+ (0.026 g, 19.5 μmol) in 2 mL of 0.1 M potassium phosphate buffer, pH 7.5. NADase (0.050 g) is added last to the mixture. The mixture is then incubated in a water-bath shaker at 37 °C for 12 hr. After incubation, the exchange reaction slurry is centrifuged at 2700 rpm (IEC, Spinette) for 5 min. The supernate is carefully collected and passed through a 0.2 μM syringe filter (Whatman) prior to separation and characterization. Because of the length of the incubation period, the laboratory instructor needs to prepare and begin the incubations the afternoon prior to the scheduled laboratory period. The students can then start the second laboratory by centrifugation of the incubation mixture and collection of the supernatant, then directly begin the next section.

Separation and Characterization (3 hr)

Thin layer chromatography (TLC) takes approximately 1 hr for application of the 3-azido NAD^+ analog to the plate and 2 hr to run. During the “run” portion of the TLC, the IR, NMR, and GC-MS characterizations can be done on the 3-azidopyridine. This sequence utilizes student laboratory time most effectively.

Thin layer chromatography lends itself well in the laboratory classroom for separation and collection of the 3-azido NAD^+ analog. The exchange reaction liquid is applied to a preparative TLC plate (i.e., 20 × 20-cm glass, 2-mm silica with F250) and developed in 90:10 methanol/water solution. After approximately 2 hr, the plates can be removed and loosely wrapped in aluminum foil, set aside to dry overnight, then refrigerated until the next laboratory period. At the beginning of the following period, the band with the NAD^+ analog (T_f = 0.46) is scraped off

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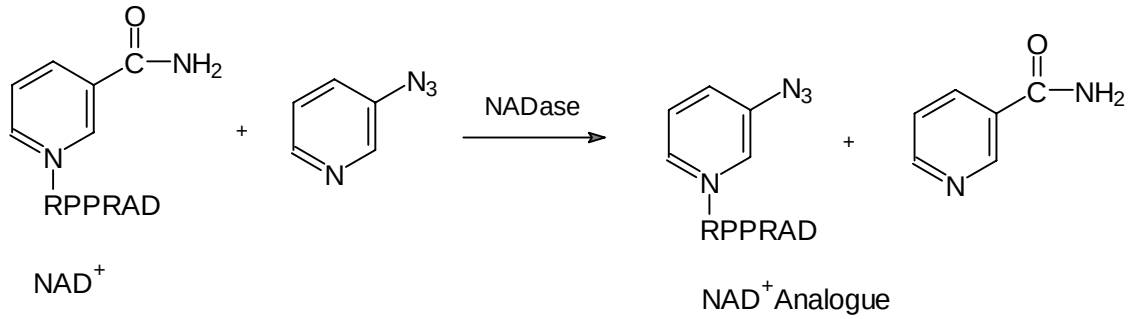

FIGURE 2. ENZYMATIC SYNTHESIS OF 3-AZIDO NAD^+ ANALOG [8].

and desorbed from the silica gel with phosphate buffer (4×5 mL) and centrifuged as before; then the supernate is collected. Typical recovery results in 20 mL of derivative with a concentration of 1.1 to 1.5×10^{-4} M. The concentration of the 3-azido NAD^+ analog in the supernate can be determined by a UV-vis scan and according to Beer's Law: absorbance = εbc, where ε_{260} = log 4.2.

Enzyme Activity Tests (Two 3-hr periods)

Preparation of the 3-azido NAD^+ analog (scraping and desorption, etc., from the TLC plate), running a positive control experiment on the 3-azido NAD^+ analog, and preparation of an NADH calibration curve will consume a 3-hr laboratory period. Competitive inhibition and irradiation experiments can be accomplished during the second 3-hr period. Because students typically outnumber instruments and cuvettes, teams can be formed. Each team can be responsible for a portion of the rate data, which is then made available to everyone.

The first enzyme activity test (positive control) normally performed with an NAD^+ analog is to determine if it is a substrate in a known enzyme-NAD^+ model system: YADH, NAD^+, and ethanol. The analog is substituted for NAD^+, and the reduction of the analog is monitored by observing any peak growth over time at 340 nm (ε_{max} NADH) by UV spectroscopy. Should there be no apparent reduction evident, then further testing is performed to determine if it is a competitive inhibitor (and useful photoaffinity label) utilizing a Lineweaver-Burke plot. Labeling efficiency can be demonstrated by comparing reaction rates with and without irradiation.

The YADH-catalyzed oxidation of ethanol by NAD^+ at room temperature serves as a suitable positive control to evaluate the ability of the 3-azido NAD^+ analog to

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replace NAD^+ as a coenzyme. Both reaction mixtures can be prepared in typical quartz UV cuvettes, 1-cm pathlength (3.5 mL). Both contain 1.5 mL of 0.05 M potassium phosphate buffer, pH 7.5; 0.5 mL of 0.05 M ethanol; 40 μL YADH (1 mg/mL). The control will contain 1.0 mL of 6×10^{-4} M NAD^+ and the other cuvette 1.0 mL of the 3-azido NAD^+ analog. Most UV instruments contain kinetic software or some means to take sequential scans at time intervals that can be set by the operator. In any case, taking scans every 20 s over a 10-min period will provide enough data to show that the 3-azido NAD^+ analog is *not* reduced, as evidenced by the absence of peak growth at 340 nm.

An NADH calibration curve is needed to convert absorbance measurements into the molar amounts. Such conversions of UV absorbance data to moles of NADH are required for construction of Lineweaver-Burke plots. Solutions of NADH spanning the concentration range of 2.5×10^{-5} to 2×10^{-4} M should be adequate to cover the absorbance recorded during the enzyme activity/irradiation tests.

Competitive Inhibition and Irradiation (3 hr)

Demonstrating that the 3-azido NAD^+ analog is a competitive inhibitor can be accomplished by constructing a Lineweaver-Burke Plot. Two sets of reaction rate (velocity) data need to be collected, one set containing NAD^+ and one set containing both NAD^+ and the 3-azido NAD^+ analog. Each set consists of five different volumes of the substrate, NAD^+: 50 μL, 100 μL, 0.3 mL, 0.6 mL, and 0.8 mL (resultant concentrations in the cuvette should bracket the concentration of 3-azido NAD^+ analog). A constant amount (40 μL) of enzyme (1 mg/mL) is added to each. For the rate-data set of the NAD^+ and 3-azido NAD^+ analog, 1.0 mL of the 3-azido NAD^+ analog is added to each cell with a corresponding reduction in the buffer added, keeping the total volume to 3 mL. Ethanol is added to the reaction mixtures last to initiate the reaction. A UV scan should be taken immediately and every 20 s for approximately 10 min.

Upon irradiation, the azido group forms a highly reactive nitrene intermediate that can insert into nearby chemical groups forming a stable bond. Formation of such a bond would irreversibly inhibit YADH active-site function and result in an overall decrease in reaction rate. This can be demonstrated by a third set of reactions using the same proportions and procedures as above, but in which the YADH and 3-azido NAD^+ analog are irradiated together for 15 s (porcelain wells on ice, 254

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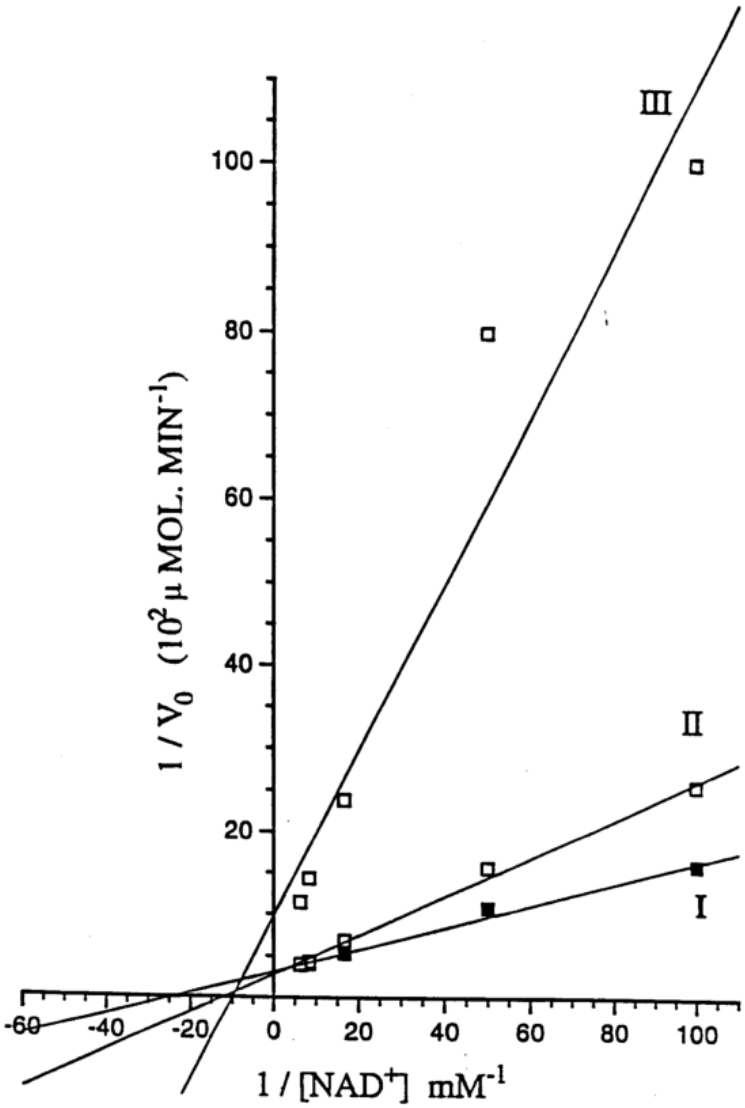

FIGURE 3. LINEWEAVER-BURKE PLOT OF ENZYME ACTIVITY TESTS DISPLAYING THE NAD^+ STANDARD **I**, INHIBITION OF YADH BY THE 3-AZIDO NAD^+ ANALOG **II**, AND THE EFFECT OF IRRADIATION WITH 254 NM LIGHT ON THE SAME **III**.

nm lamp held 10-cm from wells) prior to their addition to the cuvettes containing of the NAD^+ and buffer. Ethanol is again added to start the reaction.

Lineweaver-Burke Plot

Commercial computer programs, such as Origin or Quattro Pro, enable generation of the Lineweaver-Burke plots in a straight-forward manner. Rate data (UV absorbance, time in seconds) can be entered into data tables and then converted to

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moles/min using the NADH calibration data. NAD^+ concentration can be entered for each of the five cells; the reciprocals of the rate data (*y*-axis) and NAD^+ concentrations (*x*-axis) can be calculated and plotted (Figure 3). The slope of the lines from the NAD^+ **I** and analog **II** should be different but both should intersect the *y*-axis at the same point. The increased slope of the analog **II** results from the inhibitory effect on the reaction rates at low NAD^+ concentrations (points farthest from the origin of the graph). This effect diminishes as the concentration of NAD^+ increases. Eventually, all of the active sites are effectively occupied by NAD^+ (points closest to the origin of the graph), resulting in the same reaction rates as without the inhibitor present (common *y*-axis intersection). This demonstrates that the 3-azido analog is a competitive inhibitor. The effect of photolysis **III** can readily be seen as well by a significant velocity decrease.

Conclusion

The design of this project is intended to allow students to explore the selection and integration of chemical and instrumental techniques while improving their mastery of each. At the same time, alternative techniques are available at nearly each step to match the laboratory resources at most institutions. Each instructor's customization provides the ultimate educational benefit to the students.

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