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N-Acylethanolamines as Novel Alcohol Dehydrogenase 3 Substrates

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N-Acylethanolamines as Novel Alcohol Dehydrogenase 3 Substrates

by

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A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
Department of Chemistry
College of Arts and Sciences
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I would like to dedicate this work to my parents, Ljubica and Milorad, and my brother, Goran. Without their love and support I would not be where I am today.
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Table of Contents

List of Tables .................................................................................................................... iii
List of Figures ................................................................................................................... iv
List of Schemes ................................................................................................................ vi
Abstract ......................................................................................................................... vii
Chapter 1 - Introduction.....................................................................................................1
  N-Acylethanolamines (NAEs) ..................................................................................1
  Alcohol Dehydrogenases (ADHs) ............................................................................4
    Alcohol Dehydrogenase 3 (ADH3) .............................................................6
Chapter 2 – Material and Methods.......................................................................................9
  Enzyme Purification..................................................................................................9
    A. Ammonium Sulfate Precipitation............................................................9
    B. DEAE-Cellulose Chromatography........................................................10
    C. Mimetic Blue II Chromatography.........................................................10
    D. Blue Dextran Agarose Chromatography...............................................10
    E. Enzyme Activity Assay .........................................................................11
  Syntheses.................................................................................................................11
    A. Synthesis of NAEs ................................................................................11
    B. Synthesis of [1’,2’-13C]-N-Octanoylethanolamine................................12
    C. Synthesis of N-Acylglycinals .................................................................13
    D. Synthesis of N-Octanoylglycine............................................................14
    E. Preparation of N-Octanoylglycinal semicarbazone...............................15
  Kinetic Assays of NAE Substrate Activity with ADH3 .........................................15
    A. Spectrophotometric NADH Assay........................................................15
    B. MTS-Formazan NADH Assay..............................................................15
  Modeling Experiments............................................................................................16
List of Tables

Table 1 – Kinetic Constants for Short- and Medium-chain NAEs .................................23
Table 2 – Relative Rates for Medium- and Long-chain NAEs....................................23
Table 3 – Kinetic Constants for Aromatic NAEs and Cinnamyl Alcohol..................24
Table 4 – Relative Binding Energies and $K_M$ Values for Selected NAEs and Cinnamyl Alcohol ........................................................................................................25
List of Figures

Figure 1 – NAE Biosynthesis Reaction ...............................................................................2
Figure 2 – NAE Degradation Pathway ................................................................................2
Figure 3 – Proposed Pathway for Biosynthesis of PFAMs from NAEs ..............................3
Figure 4 – Aldehyde Dismutation Reaction .....................................................................5
Figure 5 – Formaldehyde Detoxification Pathway ..............................................................6
Figure 6 – ADH3 Dimer ......................................................................................................7
Figure 7 – MTS-Formazan Assay for NADH Detection ...................................................16
Figure 8 – SDS-PAGE of Purified Bovine Liver ADH3 .......................................................21
Figure 9 – A Representative Michaelis-Menten Plot for One of the Assayed NAEs .......22
Figure 10 – N-Hexanoylethanolamine Docked into the Active Site of Human ADH3 ....25
Figure 11 – HPLC Separation of ADH3-catalyzed N-Benzoylethanolamine Oxidation ..........................................................................................................................26
Figure 12 – HPLC Separation of ADH3-catalyzed Cinnamyl Alcohol Oxidation ..........27
Figure 13 – GC-MS Spectrum of Cinnamyl Aldehyde O-PFB-oximes .............................28
Figure 14 – GC-MS Spectrum of N,O-di-TMS-N-Benzoylglycine ..................................29
Figure 15 – Semicarbazone Formation Reaction ...............................................................30
Figure 16 – HPLC Separation of ADH3-catalyzed N-Benzoylethanolamine Oxidation in the Presence of Semicarbazide ................................................................. 30
Figure 17 – 13C NMR Spectrum of ADH3-catalyzed [1′,2′-13C]-N-Octanoylethanolamine Oxidation in the Presence of Semicarbazide .................................................31
Figure 18 – 13C NMR Spectrum of ADH3-catalyzed [1′,2′-13C]-N-Octanoylethanolamine Oxidation without Semicarbazide .................................................................32
Figure A-1 – 1H and 13C Spectra of N-Butyrylethanolamine in DMSO-d6 .....................40
Figure A-2 – 1H and 13C Spectra of N-Hexanoylethanolamine in DMSO-d6 ...............41
Figure A-3 – $^1$H and $^{13}$C Spectra of N-Octanoylthanolamine in DMSO-d$_6$ .....................42
Figure A-4 – $^1$H and $^{13}$C Spectra of N-Decanoylthanolamine in DMSO-d$_6$ ..................43
Figure A-5 – $^1$H and $^{13}$C Spectra of N-Lauroylethanolamine in DMSO-d$_6$ .................44
Figure A-6 – $^1$H and $^{13}$C Spectra of N-Myristoylethanolamine in DMSO-d$_6$ ............45
Figure A-7 – $^1$H and $^{13}$C Spectra of N-Oleoylthanolamine in DMSO-d$_6$ .................46
Figure A-8 – $^1$H and $^{13}$C Spectra of N-Benzyloylethanolamine in DMSO-d$_6$ ...........47
Figure A-9 – $^1$H and $^{13}$C Spectra of [1',2'-$^{13}$C]-N-Octanoylthanolamine in DMSO-d$_6$ .48
Figure A-10 – $^1$H and $^{13}$C Spectra of N-Benzoylglycinal in CDCl$_3$ ..........................49
Figure A-11 – $^1$H and $^{13}$C Spectra of N-Octanoylglycinal in CDCl$_3$ ...........................50
Figure A-12 – $^1$H and $^{13}$C Spectra of N-Octanoylthanolamine semicarbazone in
DMSO-d$_6$ ...........................................................................................................51
Figure A-13 – $^1$H and $^{13}$C Spectrum of N-Octanoylglycine in DMSO-d$_6$ ......................52
Figure A-14 – $^{13}$C Spectrum of [1',2'-$^{13}$C]-N-Octanoylthanolamine Control Sample
without ADH3......................................................................................................53
Figure B-1 – HPLC Separation of N-Benzylethanolamine and its Derivatives ...............54
Figure B-2 – HPLC Separation of Cinnamyl Alcohol and its Derivatives .....................55
Figure B-3 – HPLC Separation of Semicarbazide and N-Octanoylglycinal
semicarbazone...................................................................................................55
Figure C-1 – GC-MS Spectra of Cinnamyl Aldehyde O-PFB-oxime Standard ...............56
Figure C-2 – GC-MS Spectra of TMS-derivatized N-Benzoylglycine Standard ..........57
Figure C-3 – GC-MS Spectrum of N-Benzoylglycinal O-PFB-oxime Standard ..........58
List of Schemes

Scheme 1. Synthesis of NAEs ........................................................................................................12
Scheme 2. Synthesis of [1’,2’-13C]-N-Octanylethanolamine...........................................12
Scheme 3. Synthesis of N-Acylglycinals ...........................................................................14
Scheme 4. PFBHA Derivatization Reaction..........................................................................18
Scheme 5. BSTFA Derivatization Reaction .......................................................................18
N-Acylethanolamines (NAEs) are a class of fatty acid amides that act as important mammalian signaling molecules. *N*-Arachidonoylethanolamine is the best-studied representative and is one of the endogenous ligands for endocannabinoid receptors. NAEs play a role in the regulation of appetite, act as anti-inflammatory and analgesic agents, and are thought to have a neuroprotective function as well. They have been proposed to also serve as precursors to *N*-acylglycines (NAGs). *N*-Acylglycinals are likely to be intermediates between the NAEs and the NAGs. The sequential actions of a putative fatty alcohol dehydrogenase and a putative fatty aldehyde dehydrogenase are thought to affect the NAD$^+$-dependent oxidation of the NAEs to the NAGs. NAGs, in turn, serve as precursor in the biosynthesis of primary fatty acid amides (PFAMs), another class of mammalian regulatory molecules. Alcohol dehydrogenase 3 (ADH3), an enzyme known to oxidize mid- and long-chain alcohols to aldehydes, was evaluated for its potential in oxidation of NAEs to *N*-acylglycinals.

In order to evaluate the possibility of ADH3 involvement in NAE metabolism, variable chain length NAEs were synthesized and evaluated as substrates for bovine liver ADH3. NAEs were oxidized by ADH3 in the presence of NAD$^+$, yielding the corresponding *N*-acylglycinals. $V_{\text{Max}}/K_M$ values for assayed NAEs were low relative to cinnamyl alcohol, one of the preferred substrates for ADH3. Our data suggest that the ADH-mediated oxidation of NAEs could occur *in vivo*, but that ADH3 is unlikely to be the *in vivo* catalyst.
Chapter I – Introduction

_N-Acylethanolamines (NAEs)_

_N-Acylethanolamines_ (NAEs) belong to the class of fatty acid amides, which also includes primary fatty acid amides (PFAMs), _N_-acyldopamines (NADAs), _N_-acylamides (NAMs) and _N_-acylamino acids (NAAs) (1). They are the most widely studied group of fatty acid amides and represent an important class of mammalian signaling molecules. NAEs have been isolated from the brain as well as a variety of other tissues (1), and have been associated with a number of important processes, such as appetite control, regulation of energy metabolism, and anti-inflammatory response (1-3). _N_-Arachidonylethanolamine (anandamide) is an endogenous ligand for cannabinoid receptors (CB1 and CB2), and as a result, it is the most extensively studied of all NAEs. Anandamide plays an important role in the stimulation of appetite, reduction of body temperature and movement, and also has analgesic properties (1-4). It is thought that most of these effects of anandamide are mediated by its binding to CB1 receptor.

Other long-chain NAEs whose functions have been established also play important roles in the body. _N_-Palmitoylethanolamine is a well-known anti-inflammatory agent, and also has analgesic properties (1, 2, 5). _N_-Oleylethanolamine inhibits food intake through binding to PPARα (peroxisome proliferator-activated receptor α) and GPR119 (G-protein coupled receptor), thereby acting in opposition to anandamide (1, 3). _N_-Stearoylethanolamine exhibits similar effects as anandamide with regard to analgesia, body temperature and motility, although it is unable to bind to cannabinoid receptors (1). It has also recently been found to possess anti-inflammatory properties (6). Long-chain NAEs, including _N_-stearoylethanolamine and _N_-palmitoylethanolamine, have been thought to simulate the effects of anandamide by acting as “entourage” compounds (7-8). All long chain NAEs are degraded by fatty acid amide hydrolase (FAAH), and “entourage” NAEs compete with anandamide for binding to FAAH, thereby effectively
reducing its rate of degradation and prolonging its effects in the body. NAEs have also been postulated to have a neuroprotective action, as their levels are increased in injured neurons (4). Increase in NAE levels following cell stress and injury has also been found in other organs, such as heart and testes, suggesting they may play a similar role in these tissues as well (2).

Primary route for NAE synthesis is by the action of N-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD), which catalyzes the cleavage of N-acylphosphatidylethanolamine (NAPE) into NAE and phosphatidic acid (PA) (Fig. 1). NAE degradation to fatty acid and ethanolamine is accomplished primarily through the action of fatty acid amide hydrolase (FAAH), although another enzyme capable of doing the same chemistry, N-acylethanolamine-hydrolyzing acid amidase (NAAA), has been discovered recently (9-10) (Fig. 2). Alternatively, it has been proposed that NAEs could act as precursors in the biosynthetic pathway of PFAMs (1, 11), a related class of fatty acid amides (Fig 3).

![Figure 1. NAE biosynthesis reaction.](image1)

![Figure 2. NAE degradation pathway.](image2)
PFAMs were first identified in human plasma (12), but have since been isolated from brain and cerebrospinal fluid as well. Like NAEs, they function as signaling and regulatory molecules in the body. The best-studied PFAM is oleamide, which is important in regulation of sleep, memory, body temperature and movement (1). Other
naturally occurring PFAMs include erucamide, linoleamide and elaidamide (1). Initially proposed biosynthetic route to PFAMs was through reaction between fatty acid and ammonia, catalyzed by FAAH (13). However, although this chemistry occurs in vitro, it requires relatively high pH and high ammonia concentration, making it unlikely that it is the primary synthetic pathway in vivo. There are two other possible routes to PFAM biosynthesis. First route involves cytochrome c-catalyzed amidation of fatty acyl-CoA thioester by ammonia (1, 14-15). The other route is by the action of peptidylglycine-α-amidating monooxygenase (PAM) on N-acylglycines (NAG), resulting in production of PFAMs and glyoxylate (Fig. 3). NAGs are well established as good substrates for PAM, but their biosynthetic pathway is unclear. There is evidence that NAEs could serve as precursors to NAGs through the sequential actions of a putative fatty alcohol dehydrogenase (FADH) and a putative fatty aldehyde dehydrogenase (FALDH) (Fig. 3). Support for this pathway comes from experiments in which 14C-labeled N-arachidonoylglycine was detected in liver cells that were treated with 14C-labeled anandamide (11). These experiments strongly suggest that there is a set of liver ADH and ALDH enzymes capable of catalyzing the conversion of NAEs to NAGs.

Alcohol Dehydrogenases (ADHs)

Alcohol dehydrogenases (ADHs) comprise a family of NAD+-dependent, Zn-containing, dimeric enzymes responsible for oxidation of alcohols to aldehydes. ADH dimers are composed of identical subunits, each containing two Zn ions, one of which plays a structural role, and one which is involved in catalysis. Each subunit is composed of two domains: a coenzyme binding domain, located close to the interface with other subunit and a catalytic domain, located toward other ends of the dimer (Fig. 6). ADH enzymes are divided into six classes based on their structure, substrate preference, sensitivity to inhibitors, and electrophoretic mobility.

Class I ADH (ADH1) enzymes are expressed in all vertebrates, (16) and in humans they are present in the liver, kidney, lung, mucosa of the stomach and lower digestive tract (17). This class contains a number of isozymes, created through the different combinations of three subunits designated as α, β, and γ. Among ADHs, ADH1
enzymes have lowest $K_M$ values ($< 5\text{mM}$) and highest $k_{cat}$ values for ethanol, indicating that their primary role is metabolism of ethanol to acetaldehyde. They are also the most strongly inhibited by 4-methylpyrazole, and have the highest isoelectric point of all ADH enzymes (17). In addition to ethanol oxidation, ADH1 isozymes are also able to oxidize retinol, and possess aldehyde dismutase activity, converting aldehydes to carboxylic acids and alcohols (Fig. 4) (18).

Class II ADH (ADH2) is expressed exclusively in liver, and shares some of the characteristics with ADH1. It has a higher $K_M$ and lower $k_{cat}$ value for ethanol than ADH1, but it is likely also involved in ethanol metabolism. It is also inhibited by 4-methylpyrazole, but to a smaller extent than ADH1, and it has a lower pI than class I enzymes. Like ADH2, it is capable of utilizing retinol as a substrate, and it has been shown to have aldehyde dismutase activity (18).

Class IV ADH (ADH4) is found in the mucosa of the stomach and upper digestive tract, and therefore may play a role in the first-pass metabolism of ethanol (19). It shares many features with ADH2 – similar $K_M$ value for ethanol, similar pI, and similar level of inhibition by 4-methylpyrazole. However, its turnover values for both ethanol and retinol are higher, and it has been suggested that ADH4 is the primary enzyme for oxidation of retinol to retinal (20-22).

Class V ADH (ADH5) has been characterized on cDNA and mRNA level in humans (16, 23), but remains largely unstudied. Class VI (ADH6) has been isolated as cDNA from deer mouse and rat (16, 20, 24), but has also not been investigated to greater effect. The functions of these classes therefore remain largely unknown.

![Aldehyde dismutation reaction](image)

**Figure 4. Aldehyde dismutation reaction.**

Class IV ADH (ADH4) is found in the mucosa of the stomach and upper digestive tract, and therefore may play a role in the first-pass metabolism of ethanol (19). It shares many features with ADH2 – similar $K_M$ value for ethanol, similar pI, and similar level of inhibition by 4-methylpyrazole. However, its turnover values for both ethanol and retinol are higher, and it has been suggested that ADH4 is the primary enzyme for oxidation of retinol to retinal (20-22).

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5
Alcohol Dehydrogenase 3 (ADH3)

Class III alcohol dehydrogenase (ADH3) is found in virtually all eukaryotes and prokaryotes and represents the ancestral enzyme from which all the other ADHs originated. It was first isolated from human liver in 1981 (25), and it was fully characterized in 1984 (26). Shortly thereafter, it was recognized that ADH3 is identical to the previously characterized glutathione-dependent formaldehyde dehydrogenase (27-28), which catalyzes the oxidation of S-(hydroxymethyl)-glutathione (HMGSH) (formed through non-enzymatic conjugation of formaldehyde to glutathione (GSH)) (Fig. 5). Together with S-formylglutathione hydrolase, ADH3 forms a major formaldehyde detoxification pathway in the body (Fig 5).

\[
\begin{align*}
\text{HCHO} + \text{GSH} & \rightleftharpoons \text{GS-CH}_2\text{OH} & \text{ADH3} \\
& \downarrow \text{NAD+} & \uparrow \text{NADH} \\
\text{GS-CHO} & \uparrow \text{ADH3} & \downarrow \text{S-formylglutathione} \\
\end{align*}
\]

\[\text{S-formylglutathione hydrolase} \]

\[
\text{GS-CHO} \rightarrow \text{HCOOH} + \text{GSH} \]

Figure 5. Formaldehyde detoxification pathway.

Ethanol is a very poor substrate for ADH3, and saturation is never observed, precluding the determination of \(K_M\) and \(V_{\text{Max}}\) values. Unlike all the other characterized ADHs, ADH3 is not inhibited by 4-methylpyrazole, and cannot oxidize retinol. It also lacks the aldehyde dismutase activity of ADH1 and ADH2 (18). Besides HMGSH, the best substrates for ADH3 are medium- and long-chain alcohols (5 carbons and up), and \(\omega\)-hydroxy fatty acids, although it also able to oxidize 20-hydroxyeukotriene B4 (29).

This preference for larger substrates can be explained by the differences in the active site size and structure between ADH3 and other ADH enzymes characterized to date. In all ADH enzymes, active site is positioned in the cleft between the coenzyme binding domain and the catalytic domain of the subunit (Fig. 6). In ADH1 and ADH4,
binding of the coenzyme induces a conformational change whereby catalytic unit rotates by \(\sim 10^\circ\) toward the coenzyme-binding domain, thereby converting the enzyme from the “open” to the “closed” conformation (30-35). Closure of the active site enables tighter binding for the smaller substrates, accounting for the preference these enzymes have for shorter alcohols. Because NAD\(^+\) binding is required in order to achieve effective substrate binding, these enzymes display an ordered bi-bi mechanism (31-32, 36).

In contrast, the ADH3 active site displays a “semi-open” configuration that is in-between the fully “open” and fully “closed” conformations exhibited by ADH1 (30-32). In addition to this, coenzyme binding results in only small conformational changes between the two domains, leaving a large, solvent-exposed active site approximately twice the size of the ADH1 active site (30). Substrate binding is therefore not dependent on prior NAD\(^+\) binding, and, as a result, ADH3 has a random bi-bi mechanism (36). Having a large active site means that ADH3 is capable of accommodating bulky substrates, like long chain alcohols, \(\omega\)-hydroxy fatty acids and HMGSH. It also accounts for its poor activity toward the smaller substrates, such as ethanol, which are unable to form the bonding interactions they make in other ADHs. In addition to the open active site, ADH3...
site, smaller substrates lack the stabilization provided by the loop formed by residues 112 to 120 in ADH1, which is replaced by an α-helix in ADH3 which fails to provide any binding support (30).

The well-established preference of ADH3 for large, hydrophobic alcohol substrates suggests that it could act as the putative FADH, catalyzing the oxidation of NAEs to N-acylglycinals. It is also the most ubiquitous of all the ADH enzymes, and it is the sole ADH representative so far characterized in the brain (37), where abundance of NAEs is found. Its substrate preference combined with its presence in the tissues where NAEs have been found, led us to investigate ADH3 as a potential FADH, acting in the first step of the proposed PFAM biosynthetic pathway to catalyze the oxidation of NAEs to N-acylglycinals (Fig. 3).
Chapter 2 – Materials and Methods

Enzyme Purification

Bovine liver ADH3 was purified following the modified procedure of Pourmotabbed et al. (38). Purification was accomplished by performing ammonium sulfate precipitation, followed by DEAE-cellulose, mimetic blue II, and blue dextran agarose chromatography.

A. Ammonium Sulfate Precipitation

Fresh bovine liver was obtained from Central Packing Co Inc, (Center Hill, FL), cut into parts, and frozen at – 80 °C. Bovine liver portion weighing 265 g was thawed out, chopped into small pieces, combined with 795 ml of 50 mM potassium phosphate pH 7.5, 5 mM β-mercaptoethanol (BME), and a cocktail of protase inhibitors (1µg/ml leupeptin, 1 µg/ml pepstatin A, and 1 µg/ml aprotinin), and blended for 3 x 30 seconds in a Waring blender at high setting. Blended mixture was centrifuged in a Sorvall RC 6 centrifuge at 20,000 x g and 4 °C for 40 minutes. Pellet was then discarded and supernatant centrifuged at 32,000 x g and 4 °C for 30 minutes. Reddish-brown supernatant (670 ml) was obtained following the second centrifugation, and taken to 45% saturation by addition of 173 g of ammonium sulfate. Finely ground ammonium sulfate was added slowly over a 1-hour period with continuous stirring, and pH of the solution was maintained at 7.4 by addition of concentrated NH₄OH. Following salt addition, solution was left to equilibrate for 2 hours without stirring. Equilibrated solution was centrifuged at 47,800 x g for 30 minutes. Recovered supernatant (600 ml) was then taken to 67% saturation by addition of 84 g of ammonium sulfate. As previously, ammonium sulfate was added slowly over 1 hour and pH was maintained by addition of concentrated NH₄OH. Following equilibration, solution was centrifuged at 47,800 x g for 30 minutes. Supernatant was discarded and pellet dissolved in minimum amount of 10 mM Tris pH
7.6 and 5 mM BME solution. Sample was then dialyzed in the same buffer in order to remove the salt.

B. DEAE-Cellulose Chromatography

Following dialysis, sample was loaded onto a DEAE cellulose column (2 x 40 cm) previously equilibrated with 10 mM Tris pH 7.6 and 5 mM BME. Column was washed with running buffer until $A_{280}$ was reduced to 0.2, and eluent was collected in 15 ml fractions. ADH3 was eluted off the column by running a linear pH gradient with 500 ml of running buffer (10 mM Tris pH 7.6) and 500 ml of 10 mM Tris pH 7.0. Fractions were assayed for protein content by measuring absorbance at 280 nm, while enzyme activity was assayed by following NADH production using cinnamyl alcohol as substrate. Fractions with highest specific activity were pooled together and dialyzed into solution of 10 mM Tris pH 8.0 and 2 mM dithiothreitol (DTT).

C. Mimetic Blue II Chromatography

Dialyzed sample was loaded onto a Mimetic Blue II affinity column (1.5 x 20 cm) that was previously equilibrated with 10 mM Tris pH 8.0 and 2 mM DTT. Column was washed with the same buffer and flow rate was adjusted to 1 ml/min. Eluent was collected in 4 ml fractions. Protein content was monitored by measuring absorbance of fractions at 280 nm. Once $A_{280}$ was below 0.2, a linear gradient of 0-20 mM NAD$^+$ (total volume 200 ml) was applied to the column in order to elute ADH3. Fractions were assayed for enzyme activity by following NADH production at 340 nm, with cinnamyl alcohol as substrate. Fractions with highest specific activity were pooled together and dialyzed into 0.5 mM Tris pH 7.0 and 5 mM BME.

D. Blue Dextran Agarose Chromatography

Following dialysis to remove NAD$^+$, sample was loaded onto a Blue Dextran agarose column (5 ml), that had been equilibrated with 0.5 mM Tris pH 7.0 and 5 mM BME. Column was washed with the same buffer and the eluent was collected in 3 ml fractions, which were assayed for protein content and enzymatic activity as described before. Fractions with the highest specific activity were analyzed for purity using SDS-
PAGE, and those containing only a single protein band corresponding to the weight of an ADH3 subunit (~40 kDa) were pooled together. Protein concentration of purified ADH3 was determined using the Bradford assay, and concentrated enzyme was stored at -80 °C.

E. Enzyme Activity Assay

Enzyme activity was evaluated by following NADH production at 340 nm, with cinnamyl alcohol as the substrate. Assays were done in 100 mM glycine pH 10, 5 mM cinnamyl alcohol, and 2.5 mM NAD\(^+\) at 37 °C. NADH production was followed at 340 nm using JASCO V-530 UV-Vis spectrophotometer. When scanning crude enzyme fractions, which are known to include ADH1 and ADH2, assay also included 3 mM pyrazole, a known inhibitor of these two enzymes. In addition to this, rates for controls without the substrate were obtained and subtracted from the reaction rates in order to eliminate false positives.

Syntheses

A. Synthesis of NAEs

A number of N-acyl ethanolamines were available commercially in high purity and these were obtained and used without further purification. N-Arachidonoylethanolamine and N-linoleoylethanolamine were purchased from Cayman Chemical Company, N-acetylenoethanolamine was obtained from Sigma-Aldrich, and N-propionylethanolamine was purchased from TCI.

The remaining N-acyl ethanolamines were synthesized as described in Jonsson et al. (7-8) (Scheme 1). Briefly, an excess of ethanolamine (~tenfold) was added to 15-20 ml of anhydrous CH\(_2\)Cl\(_2\) and placed in an ice-bath. Acyl chloride (0.012 moles) was added drop-wise to the solution while stirring and under N\(_2\). Upon complete addition of the chloride, reaction mixture was removed from ice and left stirring at room temperature for another 12 hours. Mid- and long-chain NAEs (octanoyl and longer) were purified by recrystallization in ethanol/water. Short-chain NAEs (butyryl, hexanoyl and benzoyl) were purified using a silica column run in ethyl acetate: methanol (80:20). The yields for the synthesized NAEs were as follows: N-butyrylethanolamine – 48.3 %, N-
hexanoylethanolamine – 20.6 %, N-benzyolethanolamine – 28.2 %, N-octanoylethanolamine – 44.5 %, N-decanoylethanolamine – 77.4 %, N-lauroylethanolamine – 77.6 %, N-myristoylethanolamine – 73.4 %, N-oleoylethanolamine – 84.5 %. Purity and identity of synthesized NAEs were confirmed by $^1$H and $^{13}$C NMR on iNOVA 400 MHz (see Figures A1-A8). All acyl chlorides and ethanolamine were obtained from Sigma-Aldrich, and used without further purification. Anhydrous CH$_2$Cl$_2$ was purchased from EMD.

![Scheme 1. Synthesis of NAEs.](image)

B. Synthesis of $[^1\text{C},2^\text{13C}]$-N-Octanoylethanolamine

Uniformly $^{13}$C-labeled ethanolamine HCl was dissolved in CH$_3$CN and a 10-fold molar excess of triethylamine (TEA) under N$_2$. One equivalent of octanoyl chloride was added drop-wise, and the reaction mixture was left to stir at room temperature for 12 hours (Scheme 2). Reaction progress was monitored by TLC (ethyl acetate: methanol (80:20 (v/v))). Once reaction was > 90% complete, solvent was removed, and residue was redissolved in the same mobile phase used for TLC. $[^1\text{C},2^\text{13C}]$-N-Octanoylethanolamine was purified by silica column chromatography in 53 % yield, and characterized by $^1$H and $^{13}$C NMR spectra obtained on the Varian iNOVA 400 (Fig. A-9).

![Scheme 2. Synthesis of $[^1\text{C},2^\text{13C}]$-N-Octanoylethanolamine. $^{13}$C labeled atoms are marked by *.](image)
C. Synthesis of N-Acylglycinals

*N*-Benzoylglycinal and *N*-octanoylglycinal were prepared using the modified procedure of Brown (39) (Scheme 3). Aminoacetaldehyde diethyl acetal (50 mmoles) was dissolved in 75 ml of diethyl ether and placed in an ice-bath. Next, one equivalent (5 ml of 15 M) KOH was added to the solution. One equivalent of acyl chloride and another two equivalents of KOH were simultaneously added drop-wise to the reaction mixture. Once addition was complete, reaction was left stirring on ice for another 3 hours. Reaction progress was monitored by TLC (hexanes: ethyl acetate (1:1)). Ether and aqueous layers were separated, and ether layer was washed with 5 x 30 ml of saturated NaCl, and dried with MgSO₄. Solvent was removed under vacuum to obtain *N*-acylglycinal diethyl acetal. Product purity and identity were confirmed by ¹H and ¹³C NMR on Burker DPX 250.

*N*-Benzoylglycinal and *N*-octanoylglycinal were obtained by acid-catalyzed deprotection of the corresponding *N*-acylglycinal diethyl acetals. *N*-acylglycinal diethyl acetal was dissolved in 25 ml of diethyl ether, 10 ml of saturated NaCl, 7 ml of deionized H₂O, and 3 ml of concentrated HCl. Reaction mixture was left stirring for 12 hours, and reaction progress was monitored by TLC (CH₂Cl₂: EtOAc (80:20)). Organic layer was separated and dried with MgSO₄. Solvent was removed under vacuum and the residue was dissolved in CH₂Cl₂: EtOAc (80:20), and applied to a silica column (5 x 20 cm). *N*-Benzoylglycinal and *N*-octanoylglycinal were obtained in 9 %, and 11 % yields, respectively. Purity and identity of both compounds were confirmed by ¹H and ¹³C NMR spectra obtained on Varian iNova 400 MHz (Figs A-10-11).
D. Synthesis of N-Octanoylglycine

*N*-Octanoylglycine was synthesized following the modified procedure of Iyer et al. (40). Glycine (0.02 mol) was dissolved in 100 ml of deionized water, and pH was adjusted to 10 by addition of 2 M NaOH. One equivalent of octanoyl chloride was added drop-wise to the solution, while maintaining pH 10 with addition of 2 M NaOH. Following 1.5 hours of reaction time, solution was acidified to pH 1 by addition of 30% H$_2$SO$_4$. White precipitate formed following acidification, and was filtered off and dried. Precipitate was then recrystallized with EtOAc/petroleum ether to obtain the pure product.
in 49% yield. N-Octanoylglycine identity and purity was confirmed by $^1$H and $^{13}$C NMR on Varian iNOVA 400 (Fig A-13).

E. Preparation of N-Octanoylglycinal semicarbazone

N-Octanoylglycinal semicarbazone standard was prepared by reacting N-octanoylglycinal with a large excess (~10 fold) of semicarbazide in aqueous solution. N-Octanoylglycinal semicarbazone was purified using HPLC and characterized by $^1$H and $^{13}$C NMR on Varian iNova 400 (Fig A-12).

*Kinetic Assays of NAE Substrate Activity with ADH3*

A. Spectrophotometric NADH Assay

Kinetic constants for short and mid-length NAEs were determined from initial reaction rates obtained by following NADH production at 340 nm. Reaction conditions were as follows: 100 mM glycine pH 10, 2.5 mM NAD$^+$, and 15-30 µg/ml ADH3 in 1 ml total reaction volume. NAE concentrations were between 0.1K$_M$ and 10K$_M$. Enzyme assays were conducted at 37 °C, using JASCO V-530 UV-Vis spectrophotometer. Glycine was purchased from J.T. Baker, sodium pyrophosphate was obtained from Fisher, and NAD$^+$ was acquired from Bioworld. All were used without further purification. Rates were normalized using rate for 250 µM cinnamyl alcohol as the standard.

B. MTS-Formazan Assay

Due to the low solubility of long chain NAEs full kinetic assays to determine their kinetic constants could not be performed. As the compounds could only be assayed at relatively low concentrations (<100 µM), the rates of NADH production were very low and could not be confidently determined using the spectrophotometric NADH assay. Instead, a more sensitive method for NADH detection was employed, coupling the NADH production to the reduction of a tetrazolium dye MTS ([3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] to the
colored formazan with \( \lambda_{\max} \) at 490 nm and \( \varepsilon = 20,800 \text{ M}^{-1}\text{cm}^{-1} \) at pH 9.5 (Fig. 7). PMS (phenazinemethosulfate) was used as an intermediate electron carrier.

\[
\begin{align*}
\text{NAD}^+ & \quad \text{NADH} \\
\text{PMS}_{\text{red}} & \quad \text{PMS}_{\text{ox}} \\
\text{MTS} & \quad \text{FORMAZAN}
\end{align*}
\]

*Figure 7. MTS-formAZAN assay for NADH detection.*

NAEs with 10 or more carbon atoms in the chain were all assayed for ADH3 activity at the same concentration employing the MTS-formazan assay. The assay conditions were as follows: 100 mM sodium pyrophosphate pH 9.5, 55 \( \mu \)M NAE, 2.5 mM NAD\(^+\), 150 \( \mu \)M MTS, 8.25 \( \mu \)M PMS, 3 % DMSO and 30 \( \mu \)g/ml ADH3. Decanoyl and lauroylethanolamine were soluble enough to allow the determination of \( K_M \) and \( V_{\text{Max}} \) values as well. All reactions were done at 37 °C and the initial rates were determined by observing the increase in absorbance at 490 nm using JASCO V-530 UV-Vis spectrophotometer. MTS was purchased from Promega and PMS was obtained from TCI America. Rates for cinnamyl alcohol obtained in this manner matched the rates obtained by following NADH production at 340 nm. Rates were normalized using rate for 250 \( \mu \)M cinnamyl alcohol as the standard.

*Modeling Experiments*

The crystal structure of human ADH3 (PDB ID 1MP0 (41)) was used for grid-based ligand docking. All co-crystallized ligands deemed superfluous for enzyme function were removed from the crystal structure and polar hydrogens were added using AutoDockTools. Charges were then corrected for the requisite zinc ions and bond orders corrected for the co-substrate, NAD\(^+\). The receptor grid was prepared with a grid point
spacing of 0.2 Å using AutoGrid. The substrates of interest were then prepared using AutoDockTools to define torsions, rotamers, and polar hydrogens. The ligands were then docked into the active site of ADH using AutoDock 4.0 (42-43). All default settings were utilized with the exception of increasing the number of energy evaluations from $2.5 \times 10^4$ to $2.5 \times 10^7$.

**Product Characterization Experiments**

**A. HPLC Separation Experiments**

Experiments were performed on a HP 1100 Agilent, equipped with a 4-channel solvent mixing system, a quaternary pump, and a deuterium lamp UV detector. All separations were accomplished using a Thermo-Scientific C18 column (4.6 x 250 mm), with temperature regulated at 40 °C. *N*-Benzoylethanolamine and its derivatives were separated using either a gradient of 50 mM sodium phosphate pH 6.0: CH$_3$CN - 90: 10 to 95:5 over 20 minutes, or isocratic mobile phase of 50 mM sodium phosphate pH 6.0: CH$_3$CN (90:10 (v/v)) (Fig. B-1). Compounds were detected by following UV absorbance at 230 nm.

Cinnamyl alcohol and its derivatives were separated using gradient of 50 mM sodium phosphate pH 6.0: CH$_3$CN – 75:25 to 40:60 over 15 minutes, and compounds were detected by monitoring absorbance at 265 nm (Fig B-2).

*N*-Octanoylglycinal semicarbazone and semicarbazide were separated using gradient of H$_2$O: CH$_3$CN - 70:30 to 40:60 over 20 minutes (Fig B-3). The compounds were detected by monitoring absorbance at 210 nm.

**B. GC-MS Experiments**

GC-MS experiments were performed on Shimadzu GC-MS instrument equipped with a DB-5 (0.25 µm x 0.25 mm x 30 m) column. Compounds were extracted from reaction mixture using either ethyl ether (cinnamyl alcohol and cinnamyl aldehyde) or CH$_2$Cl$_2$ (*N*-benzoylethanolamine and its derivatives). Reaction mixture was acidified prior to *N*-benzoylglycine extraction. Solvent was removed and samples were derivatized prior to analysis in order to increase their volatility and signal strength. Aldehyde
derivatization to PFB-oximes was done by dissolving the extracted and dried residue into 90 μl of CH₃CN, adding 10 μl of 100 mM PFBHA (O-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine), and heating the solution at 60 °C for 60 minutes (Scheme 4).

Scheme 4. PFBHA derivatization reaction.

N-Benzoylglycine derivatization was done by dissolving the extracted and dried material in 100 μl of BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide), purging the solution with N₂, and heating it at 90 °C for 15-20 minutes (Scheme 5). Carboxylic group and amide moiety of NAGs can be both be derivatized by this method, as can hydroxyl and amide groups of NAEs.

Scheme 5. BSTFA derivatization reaction.
Derivatized samples (5-10 µl) were injected into the GC-MS in a splitless manner, with injection temperature at 250 °C. Temperature program was modified from Merkler et al. (44). Oven temperature was raised from 55.0 °C to 150.0 °C, at a rate of 40.0 °C/min, held at 150.0 °C for 3.6 min, then raised to 300 °C at a rate of 10.0 °C/min, and finally held at 300 °C for 1.0 min. Interface temperature between GC and MS was 280 °C, and solvent cut time was 7-9 min. For N-benzyolglycinal detection, injection volume was increased to 40 µl in order to more confidently detect the TMS-benzyolglycine derivative. Solvent cut time was raised to 11.5 min, so as to avoid column overload by the far more concentrated di- and mono-TMS-N-benzoylethanolamine derivatives eluting at 10.7 and 11.2 min, respectively. Peak identity was established by comparison of retention times and mass spectra with those of derivatized standards and library spectra.

C. Trapping N-acylglycinals with semicarbazide

In order to characterize the reaction products, N-acylglycinals, which are unstable at reaction pH, semicarbazide was employed as an aldehyde-trapping reagent. Semicarbazide was added in large excess (9-10 x substrate concentration) to the reaction mix containing 100 mM sodium pyrophosphate pH 9.5, 3-5 mM N-benzoylethanolamine, 2.5 mM NAD+, and 0.25-1.0 mg/ml ADH3. Formation of N-benzyolglycinal semicarbazone was followed by HPLC.

D. N-Acylglycinal Semicarbazone Characterization by 13C NMR

N-Octanoylglycinal semicarbazone was characterized by following the enzymatic oxidation of [1’,2’-13C]-N-octanoylethanolamine in the presence of semicarbazide by 13C NMR. The presence of N-octanoylglycinal semicarbazone was established by observing the reduction in intensity of the 13C labeled signals in the substrate and the appearance of two 13C carbon signals consistent with those of N-octanoylglycinal semicarbazone. Peak identity was confirmed by comparison with 13C spectrum of synthesized N-octanoylglycinal semicarbazone (Fig. A-12). Reaction conditions were as follows: 50 mM sodium pyrophosphate pH 8.0, 2.5 mM [1’,2’-13C]-N-octanoylethanolamine, 2.5 mM NAD+, 20 mM semicarbazide HCl, 10% D2O, and 1.7 mg/ml ADH3. Reaction mix was incubated at 37 °C, and 13C NMR spectra were taken after 24 hour and 48 hour reaction
time points. Control samples containing all the reagents except for the enzyme were handled and analyzed in the same manner. Experiments were performed on a Varian iNova 400 MHz instrument.
Chapter 3 – Results and Discussion

*ADH3 Purification*

ADH3 was purified from bovine liver to ~ 90 % homogeneity (Fig. 8), and stored at -80 °C for later use in enzymatic assays. Specific activity of purified ADH3 was 0.53 U/mg, which is somewhat lower than the previously reported values of ~ 3.0 U/mg (38). Relatively low activity is most likely due to the fact that enzyme was purified from liver that had been stored in the freezer for several months, rather than a fresh sample. Molecular weight of ADH3 subunits, calculated based on the Rf values determined from the SDS-PAGE was 38 kDa, which is in good agreement with previously determined values of 40-41 kDa (32).

![Figure 8. SDS-PAGE of purified bovine liver ADH3.](image)

The outer two lanes contain the Kaleidascope molecular weight ladder (MWL), and the inner lanes contain purified ADH3.
A variety of aliphatic NAEs with chain lengths varying from 2 to 20 carbons, as well as several aromatic NAEs were evaluated as substrates for ADH3, in the manner described previously (Fig. 9). All of the tested NAEs were found to be substrates for ADH3, with $K_M$ values decreasing with increase in chain length of the acyl substituent (Table 1). This indicated that increase in substrate size led to tighter binding to the enzyme, or rather that small size impaired the ability to bind to the site tightly, which was in agreement with known substrate preferences of ADH3 (26) and previous crystallographic studies of the enzyme active site (30-32). $V_{\text{Max}}$ followed the same trend, decreasing with increase in substrate size and hydrophobicity. However, decrease in $K_M$ was more dramatic, resulting in overall increase in $V_{\text{Max}}/K_M$ values with increase in substrate chain length (see Table 1). These findings are in agreement with the previous studies, which established the preference of ADH3 for bulky, longer chain alcohol substrates, in contrast with other ADH enzymes (26). While ADH3 could catalyze the oxidation of all tested NAEs, their turnover values were low compared to cinnamyl alcohol, one of the preferred ADH3 substrates (see Tables 1 and 3).

![Michaelis-Menten plot of ADH3-catalyzed N-hexanoylethanolamine oxidation](image)

$V_{\text{Max}} = 1.6 \pm 0.018 \mu\text{mol/min/mg}$

$K_M = 9.5 \pm 0.36 \text{mM}$

**Figure 9.** A representative Michaelis-Menten plot for one of the assayed NAEs.
Table 1. Kinetic constants for short- and medium-chain NAEs.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( R )</th>
<th>( K_M ) (mM)</th>
<th>( V_{Max} ) (µmol/min/mg)</th>
<th>( V_{Max} / K_M )</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-acetylethanolamine</td>
<td>( R = \text{CH}_3 )</td>
<td>((4.5 \pm 0.35) \times 10^2)</td>
<td>1.9 \pm 0.059</td>
<td>0.0041</td>
</tr>
<tr>
<td>N-propionylethanolamine</td>
<td>( R = \text{CH}_3\text{CH}_2 )</td>
<td>79 \pm 3.8</td>
<td>1.9 \pm 0.026</td>
<td>0.024</td>
</tr>
<tr>
<td>N-butyrylethanolamine</td>
<td>( R = \text{CH}_3(\text{CH}_2)_2 )</td>
<td>46 \pm 5.4</td>
<td>1.7 \pm 0.052</td>
<td>0.038</td>
</tr>
<tr>
<td>N-hexanoylethanolamine</td>
<td>( R = \text{CH}_3(\text{CH}_2)_4 )</td>
<td>9.5 \pm 0.36</td>
<td>1.6 \pm 0.018</td>
<td>0.17</td>
</tr>
<tr>
<td>N-octanoylethanolamine</td>
<td>( R = \text{CH}_3(\text{CH}_2)_6 )</td>
<td>5.8 \pm 0.38</td>
<td>1.5 \pm 0.028</td>
<td>0.26</td>
</tr>
<tr>
<td>N-decanoylethanolamine</td>
<td>( R = \text{CH}_3(\text{CH}_2)_8 )</td>
<td>0.32 \pm 0.029</td>
<td>0.57 \pm 0.024</td>
<td>1.8</td>
</tr>
<tr>
<td>N-lauroylethanolamine</td>
<td>( R = \text{CH}_3(\text{CH}<em>2)</em>{10} )</td>
<td>0.033 \pm 0.0061</td>
<td>0.14 \pm 0.0088</td>
<td>4.3</td>
</tr>
</tbody>
</table>

Kinetic constants could not be determined for NAEs longer than lauroyl due to very low solubility and low reaction rates. Table 2 shows a comparison of rates for two medium-chain (decanoyl and lauroyl) and several long chain NAEs at a single substrate concentration. Rates continue to follow a decreasing trend observed with short and medium chain NAEs, with the exception of myristoylethanolamine which has a significantly lower rate compared to the longer NAEs tested. It is possible that this result is related to the unsaturated bonds present in other higher length NAEs, which may enable tighter binding to the enzyme.

Table 2. Relative ADH3 rates for medium and long-chain NAEs

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( R )</th>
<th>Relative Rates</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-decanoylethanolamine</td>
<td>( R = \text{CH}_3(\text{CH}_2)_8 )</td>
<td>1.0</td>
</tr>
<tr>
<td>N-lauroylethanolamine</td>
<td>( R = \text{CH}_3(\text{CH}<em>2)</em>{10} )</td>
<td>0.57</td>
</tr>
<tr>
<td>N-myristoylethanolamine</td>
<td>( R = \text{CH}_3(\text{CH}<em>2)</em>{12} )</td>
<td>0.061</td>
</tr>
<tr>
<td>N-oleoylethanolamine</td>
<td>( R = \text{CH}_3(\text{CH}<em>2)</em>{14} )</td>
<td>0.28</td>
</tr>
<tr>
<td>N-linoleoylethanolamine</td>
<td>( R = \text{CH}_3(\text{CH}<em>2)</em>{16} )</td>
<td>0.26</td>
</tr>
<tr>
<td>N-arachidonoylethanolamine</td>
<td>( R = \text{CH}_3(\text{CH}<em>2)</em>{18} )</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Among the NAEs with an aromatic substituent, compounds with benzyl group had lower \( K_M \) values, higher \( V_{Max} \) values, and therefore higher turnover values compared to compounds with a phenyl substituent (see Table 3), indicating that the presence of \( \text{CH}_2 \)
group following the aromatic ring might have contributed to better binding and more efficient catalysis.

Table 3. Kinetic constants for aromatic NAEs and cinnamyl alcohol.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>( R )</th>
<th>( K_M ) (mM)</th>
<th>( V_{Max} ) (µmol/min/mg)</th>
<th>( V_{Max} / K_M )</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-benzoylethanolamine</td>
<td>( C_6H_6 )</td>
<td>5.2 ± 0.52</td>
<td>0.36 ± 0.0098</td>
<td>0.069</td>
</tr>
<tr>
<td>N-phenylacetylethanolamine</td>
<td>( C_6H_6CH_2 )</td>
<td>3.9 ± 0.24</td>
<td>1.2 ± 0.026</td>
<td>0.32</td>
</tr>
<tr>
<td>N-benzoxycarbonylethanolamine</td>
<td>( C_6H_6CH_2O )</td>
<td>2.3 ± 0.14</td>
<td>0.81 ± 0.014</td>
<td>0.36</td>
</tr>
<tr>
<td>N-(2-Phenoxyacetyl)ethanolamine</td>
<td>( C_6H_5OCH_2 )</td>
<td>6.3 ± 0.36</td>
<td>0.25 ± 0.0042</td>
<td>0.040</td>
</tr>
<tr>
<td>Cinnamyl alcohol</td>
<td>( C_6H_5HC=CHCH_2OH )</td>
<td>0.035 ± 0.0033</td>
<td>4.0 ± 0.058</td>
<td>4.1 × 10^4</td>
</tr>
</tbody>
</table>

Docking Experiments

NAEs with chain length between 2 and 11 carbons were docked into the human ADH3 crystal structure using AutoDock 4.0 in order to evaluate their relative binding energies (Table 4). Sequence alignment of human and bovine ADH3 using BLAST (45-46) shows that they share 94% identity, with 98% of residues conserved, making the docking results obtained with the human enzyme applicable to bovine ADH3 as well. Relative binding energies of docked NAEs were found to follow the same general trend as \( K_M \) values, decreasing with increase in chain length (Table 4), indicating that tighter binding to the enzyme is at least partly responsible for the observed \( K_M \) trend. These findings provide further agreement with previously determined substrate preference (26) and crystallographic data (30-32). Substrates were docked in the enzyme active site with hydroxyl group coordinating with the catalytic Zn ion (Fig. 7), in the same manner as other ADH3 substrates have been shown to bind (31-32).
Table 4. Relative binding energies and $K_M$ values for selected NAEs and cinnamyl alcohol. Binding energies values were calculated using Autodock 4.0, and have a standard error of about $\pm$ 2.5 kcal/mol (43).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_M$ (mM)</th>
<th>Estimated free energy of binding (kcal/mol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-acetylenlethanolamine</td>
<td>($4.5 \pm 0.35$) x $10^{-2}$</td>
<td>-3.72</td>
</tr>
<tr>
<td>N-propionyllethanolamine</td>
<td>79.3 ± 3.8</td>
<td>-4.14</td>
</tr>
<tr>
<td>N-butyrethanolamine</td>
<td>46 ± 5.4</td>
<td>-4.22</td>
</tr>
<tr>
<td>N-hexanoylethanolamine</td>
<td>9.5 ± 0.36</td>
<td>-4.34</td>
</tr>
<tr>
<td>N-octanoylethanolamine</td>
<td>5.8 ± 0.38</td>
<td>-4.31</td>
</tr>
<tr>
<td>N-decanoylethanolamine</td>
<td>0.32 ± 0.029</td>
<td>-4.34</td>
</tr>
<tr>
<td>N-lauroylethanolamine</td>
<td>0.033 ± 0.0061</td>
<td>-4.06</td>
</tr>
<tr>
<td>N-benzoylethanolamine</td>
<td>5.2 ± 0.52</td>
<td>-4.58</td>
</tr>
<tr>
<td>Cinnamyl alcohol</td>
<td>0.035 ± 0.0033</td>
<td>-4.68</td>
</tr>
</tbody>
</table>

Figure 10. N-Hexanoylethanolamine docked into the active site of human ADH3 (PDB ID 1MP0). Protein backbone is shown in green, Zn ions are gray spheres, and NAD$^+$ and N-hexanoylethanolamine are shown as stick models with CPK-colored atoms.
Characterization of the product of the enzymatic reaction was initially attempted by following the reaction progress by HPLC. HPLC analysis of the ADH3-catalyzed N-benzoylethanolamine oxidation revealed the presence of a peak consistent with N-benzyolglycine as well as a peak corresponding to N-benzyolglycinal (Fig. 11). N-Benzoylglycine and N-benzyolglycinal are the two possible products of the enzymatic oxidation of N-benzoylethanolamine. However, ADH3 has not been found to catalyze the dismutation of aldehydes (18). Experiments using N-benzyolglycinal as a substrate for ADH3 indicated that ADH3 is not able to catalyze the oxidation of N-benzyolglycinal, but that N-benzyolglycinal can undergo non-enzymatic dismutation to N-benzyolglycine and N-benzoylethanolamine under the employed reaction conditions (2.5 mM NAD\(^+\), pH 9.5-10). HPLC analysis of ADH3-catalyzed cinnamyl alcohol oxidation revealed only peak consistent with cinnamyl aldehyde (Fig. 12), further suggesting that the reaction products of NAE oxidation are N-acylglycinals, and that any NAGs observed are produced through non-enzymatic means.

![Figure 11. HPLC separation of ADH3-catalyzed N-benzoylethanolamine oxidation.](image)

Reaction conditions were as follows: 100 mM sodium pyrophosphate pH 9.5, 2.5 mM NAD\(^+\), 6 mM N-benzoylethanolamine, and 2.4 mg/ml ADH3. An aliquot was taken for HPLC analysis after 36 hours of reaction time. Peak at 3.918 was determined to be NAD\(^+\) derivative formed during prolonged incubation at high pH and was also observed in the control sample containing only NAD\(^+\) and buffer.
Further characterization of the product peaks observed by HPLC was conducted using GC-MS. In order to test the validity of the method, the procedure was first used to characterize cinnamyl alcohol reaction product. Derivatization of cinnamyl alcohol reaction extract with PFBHA gave two peaks detected by GC-MS, whose retention times and fragmentation patterns were consistent with those for two isomers of cinnamyl aldehyde PFB-oxime (Fig. 13), thus confirming that the reaction product is cinnamyl aldehyde. However, applying the same method to characterize N-benzoylglycinal observed by HPLC proved unsuccessful. Although derivatized standard for N-benzoylglycinal-PFB-oxime was successfully characterized using GC-MS (Fig C-3), the compound could not be detected by this method in the reaction extracts. This can be attributed to the very low turnover rates of N-benzoylethanolamine oxidation compared to cinnamyl alcohol oxidation (Table 3), as well as to the tendency of N-benzoylglycinal to undergo oxidation to N-benzoylglycine under the reaction conditions. Derivatizing reaction extract with BSTFA resulted in detection of N,O-di-TMS-N-benzoylglycine (Fig. 14), thus confirming that the peak observed by HPLC is N-benzoylglycine.
Figure 13. GC-MS spectra of cinnamyl aldehyde O-PFB-oximes. Cinnamyl aldehyde PFB-oximes were detected through GC-MS analysis of cinnamyl alcohol reaction extract that was treated with PFBHA. The two peaks correspond to the two cinnamyl aldehyde O-PFB-oxime isomers (E and Z), which differ in retention times, but have identical fragmentation patterns. See Fig. C-1 for cinnamyl aldehyde O-PFB-oxime standard spectra.
Figure 14. GC-MS spectrum of N,O-di-TMS-N-benzoylglycine. N,O-di-TMS-N-benzoylglycine peak was detected in derivatized extract of ADH3-catalyzed N-benzoylethanolamine reaction. Extraction and GC-MS analysis were performed after 24 hours of reaction time.

In order to detect and characterize the unstable N-acylglycinal product, a well-known aldehyde-trapping reagent, semicarbazide, was added to the reaction mixture. Semicarbazide readily reacts with aldehydes and ketones to form semicarbazones (Fig. 15). HPLC analysis of N-benzoylethanolamine reaction containing semicarbazide revealed a peak consistent with the peak for N-benzoylglycinal semicarbazone standard (Figs.12 and B-1). Further characterization of the semicarbazone derivative was attempted by LC-MS and GC-MS, but proved unsuccessful.
Figure 15. Semicarbazone formation reaction.

In order to characterize the putative N-acylglycinal semicarbazone species detected by HPLC, reaction was analyzed by $^{13}$C NMR. For the purpose of increased sensitivity, $[1',2'-^{13}$C]-$N$-octanoylthanolamine was synthesized and employed as the reaction substrate. Analysis of $[1',2'-^{13}$C]-$N$-octanoylthanolamine reaction showed a total of 4 different $^{13}$C signals – two that matched those for the substrate, at 41 and 60 ppm (Fig. A-9), and two new $^{13}$C signals, at 40 and 143 ppm. Chemical shifts of the two new signals match those of the corresponding carbon atoms in the $N$-octanoylglycinal semicarbazone standard (Figs. 17 and A-12), thus providing confirmation that the product of the ADH3 catalyzed oxidation is $N$-acylglycinal, in agreement with previously published data (18). Performing the reaction in absence of semicarbazide resulted in
appearance of $^{13}\text{C}$ signals consistent with those of $N$-octanoylglycine (Figs. 18 and A-13). This result was consistent with previous HPLC experiments, which showed that $N$-benzoylglycine is readily oxidized to $N$-benzoylglycine at basic pH. Control sample with no ADH3 present showed only the two substrate peaks (Fig A-14), confirming there was no detectable oxidation of $N$-octanoylethanolamine through non-enzymatic means.

Figure 17. $^{13}\text{C}$ NMR spectrum of ADH3-catalyzed $[1',2'-^{13}\text{C}]-N$-octanoylethanolamine oxidation in the presence of semicarbazide. Reaction conditions were as indicated in Chapter 2, and spectrum was taken after 48 hours of reaction time.
Figure 18. \(^{13}\text{C}\) NMR spectrum of ADH3-catalyzed \([1',2'-^{13}\text{C}]-\text{N-octanoylthanolamine oxidation without semicarbazide.}\) Reaction conditions were as indicated in Chapter 2 with the exception of semicarbazide, which was omitted. Spectrum was taken after 48 hours of reaction time.

**Conclusion**

Evaluation of NAEs as ADH3 substrates showed that they can be oxidized by this enzyme, which is known to act on a variety of mid- and long-chain alcohol substrates. In accordance with previous literature reports, the reaction products were shown to be \(N\)-acylglycinals, which ADH3 was unable to oxidize further to NAGs. Relatively low conversion rates observed for ADH3-catalyzed NAE oxidation make it unlikely that ADH3 plays a significant role in conversion of NAEs to \(N\)-acylglycinals *in vivo*. Nevertheless, our findings validate the chemistry proposed in the pathway, and further suggest that another ADH enzyme could be responsible for the oxidative degradation of NAEs. Further studies on ADH5 and ADH6 should be completed in order to evaluate the possibility of their involvement in NAE metabolism, as these two enzymes have as of yet been poorly investigated and could potentially play the role of FADH in the conversion
of NAEs to NAGs. Additionally, NAEs could be evaluated as substrates or inhibitors of other ADH enzymes, in order to explore their potential role in alcohol or retinol metabolism.
References Cited


APPENDICES
Figure A-1. $^1$H and $^{13}$C NMR Spectra of N-Butyrylethanolamine in DMSO-$d_6$. Peak at 3.409 ppm in the $^1$H spectrum is due to presence of a small amount of H$_2$O in the solvent.
Figure A-2. $^1$H and $^{13}$C NMR Spectra of $N$-Hexanoylethanolamine in DMSO-d$_6$. 

Appendix A (Continued)
Figure A-3. $^1$H and $^{13}$C NMR Spectra of N-Octanoylethanolamine in DMSO-d$_6$. 
Figure A-4. $^1$H and $^{13}$C NMR Spectra of N-Decanoylethanolamine in DMSO-d$_6$. 
Figure A-5. $^1$H and $^{13}$C NMR Spectra of N-Lauroylethanolamine in DMSO-d$_6$. 
Figure A-6. $^1$H and $^{13}$C NMR Spectra of N-Myristoyl ethanolamine in DMSO-d$_6$. Peak at 3.280 ppm in the $^1$H spectrum is due to presence of a small amount of H$_2$O in the solvent.
Figure A-7. $^1$H and $^{13}$C NMR Spectra of N-Oleylethanolamine in DMSO-d$_6$. 
Figure A-8. $^1$H and $^{13}$C NMR Spectra of N-Benzylethanolamine in DMSO-d$_6$. Peak at 3.398 ppm in the $^1$H spectrum is due to presence of a small amount of H$_2$O in the solvent.
Figure A-9. $^1$H and $^{13}$C NMR Spectra of [1',2'-$^{13}$C]-N-Octanoyl ethanolamine in DMSO-d$_6$. Peak at 3.287 ppm in the $^1$H spectrum is due to presence of a small amount of H$_2$O in the solvent. $^{13}$C-labeled atoms are denoted by *.
Figure A-10. $^1$H and $^{13}$C Spectra of N-Benzoylglycinal in CDCl$_3$. Peak at 1.598 ppm in the $^1$H spectrum is due to presence of a small amount of H$_2$O in the solvent.
Figure A-11. $^1$H and $^{13}$C NMR Spectra of N-Octanoylglycinal in CDCl$_3$. 
Figure A-12. $^1$H and $^{13}$C NMR Spectra of N-Octanoylglycinal semicarbazone in DMSO-$d_6$. Peak at 1.598 ppm in the $^1$H spectrum is due to presence of a small amount of H$_2$O in the solvent. Signal for C atom marked by † is obscured by the DMSO solvent peak in the $^{13}$C spectrum.
Figure A-13. $^1$H and $^{13}$C NMR Spectra of N-Octanoylglycine in DMSO-$d_6$. 

Appendix A (Continued)
Figure A-14. $^{13}\text{C}$ NMR spectrum of [$1',2'-^{13}\text{C}$]-N-octanolethanolamine control sample without ADH3.
Figure B-1. HPLC separation for N-benzylethanolamine and its derivatives. (a) Mobile phase: gradient of 90:10 (50 mM sodium phosphate pH 6.0: CH₃CN) to 95:5 over 20 minutes. (b) Mobile phase: 90:10 (50 mM sodium phosphate pH 6.0: CH₃CN).
Figure B-2. HPLC separation of cinnamyl alcohol and its derivatives.

Figure B-3. HPLC separation of semicarbazide and N-octanoylglycinal semicarbazone.
Appendix C – GC-MS Spectra

Figure C-1. GC-MS Spectra of cinnamyl aldehyde O-PFB-oxime standard.
Appendix C (Continued)

Figure C-2. GC-MS spectra of TMS-derivatized N-benzyolglycine standard.
Figure C-3. GC-MS Spectrum of N-benzyglycine O-PFB-oxime standard. E and Z isomers are not resolved.