# Enzymatic Synthesis of Caged NADP Cofactors: Aqueous NADP Photorelease and Optical Properties

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*Received November 16, 1999*

The synthesis of caged NADP analogues **18**, **19**, and **20** has been accomplished by utilizing the transglycosidase activity of solubilized NAD glycohydrolase (porcine brain) to incorporate caged nicotinamides **2**, **3**, and **4** into NADP. The synthesis of several nicotinamides modified at the carboxamide with *o*-nitrobenzyl photolabile groups is demonstrated as well as their potential for enzymatic transglycosidation. These results further demonstrate the feasibility of direct enzymatic transglycosidation of sterically hindered substrates into NAD(P), although high nicotinamide analogue water solubility was found to be a necessary trait for yield enhancement with certain analogues. Caged analogues were surveyed under aqueous conditions for net NADP photorelease, while the UV and fluorescent properties of both analogues and their photobyproducts were assessed for compatibility with systems that rely on optical monitoring of enzyme activity. A highly water-soluble α-methyl-*o*-nitrobenzyl group **8** was developed for the synthesis of **20** in order to enhance net NADP photorelease. Compound **20** demonstrated a high 75% net NADP photoreleased without substantial UV optical blackening or fluorescent byproducts. Analogues **18** and **19** were shown by ESI/MALDI-MS to photogenerate primarily adducts of NADP with deleterious UV and fluorescent properties. Our work stresses the superior release properties conferred by α-methyl substitution on aqueous carboxamide photorelease from *o*-nitrobenzyl compounds.

## Introduction

The NAD(P)-dependent oxidoreductases comprise some several hundred enzymes that are indispensable for life.[1] We have previously demonstrated the synthesis and photochemistry of *o*-nitrobenzyl-modified NAD(P) analogues that release NAD(P) upon UV irradiation.[2] The probing of biological processes with such light-sensitive "caged" compounds has proven useful in applications that range from enzyme kinetics to cellular physiology.[3] Detection of NAD(P) dependent oxidoreductase activity can be correlated with the distinctive UV and fluorescence changes that differentiate the NADP pyridine moiety from the NADPH 1,4-dihydropyridine group, Figures 1 and 2. The reversible, enzyme-triggered "switch" between the oxidized and reduced states of NAD(P) provides one of the most commonly used tools for the study of oxidoreductase enzyme kinetics as well as serving as a measure of the cellular redox state.[4] It has been our objective to design caged NADP analogues that allow the efficient exploitation of these novel optical properties. Optimal NADP photorelease from analogues accompanied by minimal optical interference from photobyproducts were thus guiding criteria.

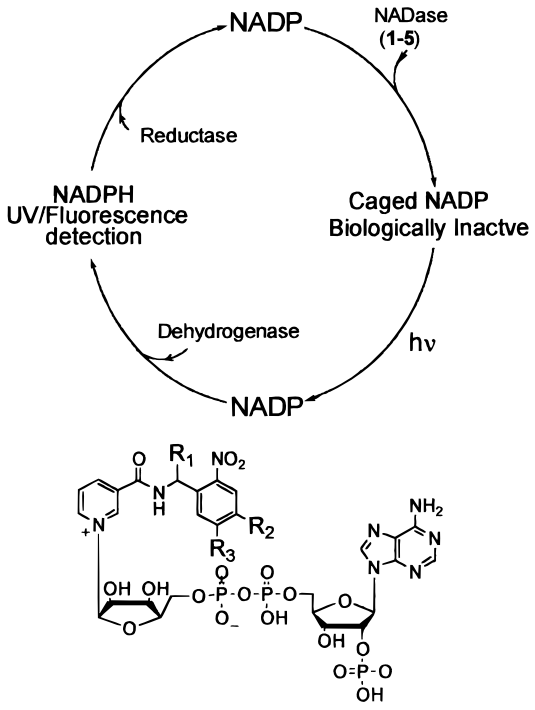


**Figure 1.** Enzymatic incorporation of caged nicotinamides (**1**−**5**) and subsequent use as detectors of oxidoreductase activity via the photorelease of NADP.

Our overall synthetic strategy for analogue synthesis has utilized the stereoselective transglycosidation activity of a commercially available glycohydrolase (NADase) to incorporate *o*-nitrobenzyl-modified nicotinamides into NADP.[2] This simple methodology[5] has also been extensively utilized to exchange a variety of modified pyridines

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(1) (a) White, H, B. In *The Pyridine Nucleotide Coenzymes*; Everse, J., Anderson, B., You, K.-SA., Eds.; Academic Press: New York, 1982; pp 4−6. (b) Basseguy, D.-R.; Bergel, A.; Comtat, M. *Enzyme Microb. Technol.* **1997**, *20*, 248.

(2) Salerno, C. P.; Resat, M.; Magde, D.; Kraut, J. *J. Am. Chem. Soc.* **1997**, *119*, 3403.

(3) Reviews on caged compounds: (a) Adams, S. R.; Tsien, R. Y. *Annu. Rev. Physiol.* **1993**, *55*, 755. (b) Kaplan, J. H. *Annu. Rev. Physiol.* **1990**, *32*, 897. (c) Givens, R. S.; Kueper, L. W. *Chemical Reviews*. **1993**, *93*, 55. (d) McCray, J. A.; Trentham, D. R. *Annu Rev. Biophys. Chem.* **1989**, *18*, 239.

(4) (a) Marose, S.; Lindemann, C.; Ulber, R.; Scheper, T. *Trends Biotechnol.* **1999**, *17*, 30. (b) Shear, J. B. *Anal. Chem*. **1999**, *71*, 598.

10.1021/jo9917846 CCC: $19.00 
Published on Web 06/08/2000

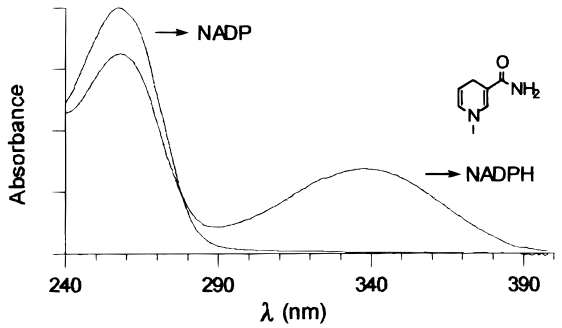


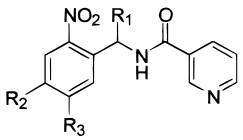

**Figure 2.** Ultraviolet spectra of NADP $\lambda_{max}$ (258 nm), $\epsilon$ = 18 000 $M^{-1}$ $cm^{-1}$ and NADPH $\lambda$ (340 nm), $\epsilon$ = 6200 $M^{-1}$ $cm^{-1}$.

both in vitro *and* in vivo.[6] The placement of the caging group at the carboxamide has the advantage of promoting biological inactivation while minimally interfering with the ADPP (2′-phosphoadenosine-5′-diphosphosphate) enzyme binding domain of NADP. This provides the opportunity for photoactivation of bound cofactor/enzyme complexes.[7]

The release properties of *o*-nitrobenzyl groups have been shown to be highly dependent upon the medium of photolysis.[3d] *O*-Nitrobenzyl groups are the only photolabile groups for which efficient release of the carboxamide group has been reported under aqueous conditions.[2,9] The few quantitative studies of carboxamide photorelease from *o*-nitrobenzyl groups were conducted using organic, mixed aqueous/organic solvent systems.[10] Our earlier study[2] demonstrated modest NADP photorelease from *o*-nitrobenzyl-caged analogue **17**, but the generation of a highly reactive *o*-nitrosoaldehyde byproduct was of particular concern for studies involving biological systems.[11] The use of α-methyl-*o*-nitrobenzyl groups was introduced by Kaplan et al.[12] as a means to ameliorate side reactions by virtue of a less reactive *o*-nitrosoketone byproduct. The use of α-methyl substitution has consistently proved capable of increasing net yields of product release as well as increasing release rates in both caged compounds and photolabile linkers.[8,13] We will demonstrate the synthesis of a novel α-methyl-*o*-nitrobenzyl group and its NADase facilitated incorporation into NADP. The photochemical release properties and optical assessment of several caged NADP analogues under aqueous conditions will be described as well as the strong tendency for undesired photobyproduct formation with certain analogues.

## Results and Discussion

**Synthesis of Caged Nicotinamides.** The synthesis of caged nicotinamides **2**−**5** followed several routes which required the synthesis of the necessary *o*-nitrobenzylamines followed by efficient coupling with nicotinoyl chloride (NCl). Notably, compound **7** was synthesized via the regioselective nitration of a phthalimide-protected aromatic. Compounds **2** and **5** were synthesized via a borane reduction methodology,[2,14] while compound **4** required an updated synthesis of *o*-nitrophenyl glycine.




**1** ($R_1$ = H, $R_2$ = H, $R_3$ = H)
**2** ($R_1$ = H, $R_2$ = OMe , $R_3$ = OMe)
**3** ($R_1$ = $CH_3$, $R_2$ = $CO_2H$, $R_3$ = H)
**4** ($R_1$ = $CO_2H$, $R_2$ = H, $R_3$ = H)
**5** ($R_1$ = $CH_3$, $R_2$ = H, $R_3$ = H)

Our initial attempts at direct nitration of 4-(1-bromoethyl)benzoic acid to produce the 3-nitro bromide, Rich and Gurawa,[15] resulted in its rapid decomposition at −10 °C in fuming nitric acid. The synthesis of **8** was thus to be approached through nitration of phthalimide ester **6**, Scheme 1. Hence, 4-(1-bromoethyl)benzoic acid (Lancaster) was transformed to its ethyl ester using acetyl chloride/EtOH in 92% yield. Coupling of potassium phthalimide could only be achieved by refluxing a concentrated solution of (3:1) DMF/ester with 1 equiv of potassium phthalimide for 1 h, providing **6** in 85% yield.[16] Nitration of **6** at −10 °C in fuming nitric acid produced **7** in 71% yield with high regioselectivity.[17] Amine **8** was then coupled with NCl to produce **9**, while nicotinamide **3** was generated using mild $K_2CO_3$-induced saponification.

The synthesis of **4** required *o*-nitrophenyl glycine, first synthesized by Davis et al.[18] via $PBr_3$-catalyzed bromination of *o*-nitromandelate followed by phthalimide coupling and then acidolysis. The noncommercial availability of *o*-nitromandelate suggested an alternative synthesis of the α-benzyl bromide, Scheme 2.[19] Thus, α-bromination of methyl *o*-nitrophenylacetate using 2

(5) (a) Anderson, B. M. In *The Pyridine Nucleotide Coenzymes*; Everse, J., Anderson, B., You, K.-SA., Eds.; Academic Press: New York, 1982; pp 91−133. (b) Woenckhaus, C.; Jeck, R. In *Pyridine Nucleotide Coenzymes: Chemical, Biochemical, and Medical Aspects, Part A*; Dolphin, D., Avramovic, O., Poulson, R., Eds.; Wiley: New York, 1987; pp 449−569.

(6) (a) Shen, W. C.; Greene, K. M.; Van Vunakis, H. *Biochem. Pharmacol.* **1977**, *26*, 1841. (b) Dietrich, L. S.; Friedland, I. M.; Kaplan, N. O. *J. Biol. Chem*. **1958**, *233*, 964.

(7) (a) Ulrich, G, M. In *The Pyridine Nucleotide Coenzymes*; Everse, J., Anderson, B., You, K.-SA., Eds.; Academic Press: New York, 1982; pp 156−180. (b) For a comprehensive listing and analysis of NADP and NADP analogue binding with dihydrofolate reductase, see: Sawaya, M. R.; Kraut, J. *Biochemistry* **1997**, *36*, 586.

(8) Ramesh, D.; Wieboldt, R.; Billington, A. P.; Carpenter, B. K.; Hess, G. P. *J. Org. Chem*. **1993**, *58*, 4599.

(9) It is commonly found in the literature of caged compounds that *net* yields of photoconversion are absent. Quantum efficiencies by themselves are not necessarily reflective of the actual net product release yield. Quantum efficiencies for the appearance of substrates are typically calculated under conditions which attempt to minimize the net photoconversion to product(s) (1−5%). Under such conditions it is difficult to assess for byproduct formation or extrapolate accurately to a net product yield.

(10) (a) Henriksen, D. B.; Breddam, K.; Moller, J.; Buchardt, O. *J. Am. Chem. Soc.* **1992**, *114*, 1876. (b) Henriksen, D. B.; Breddam, K.; Buchardt, O. *Int. J. Peptide Protein Res.* **1993**, *41*, 169. (c) Hammer, R. P.; Albericio, F.; Gera, L.; Barany, G. *Int J. Peptide Protein Res.* **1990**, *36*, 31. (d) Rich, D. H.; Gurawa, S, K. *Tetrahedron Lett.* **1975**, *5*, 301.

(11) (a) Barth, A.; Corrie, J. E. T.; Gradwell, M. J.; Maeda, Y.; Mantele, W.; Meier, T, Trentham, D. R. *J. Am. Chem. Soc.* **1997**, *119*, 4149. (b) Pirrung, M. C.; Lee, Y. R.; Park, K.; Springer, J. B. *J. Org. Chem*. **1999**, *64*, 5042.

(12) Kaplan, J. H.; Forbush, B.; Hoffman, J. J. *Biochemistry* **1978**, *17*, 1929.

(13) (a) Sternson, S. M.; Schreiber, S. L. *Tetrahedron Lett.* **1998**, *39*, 7451−7454. (b) Holmes, C. P.; Jones, D. G. *J. Org. Chem*. **1995**, *60*, 2318.

(14) Feuer, H.; Braunstein, D. M. *J. Org. Chem*. **1969**, *34*, 1817.

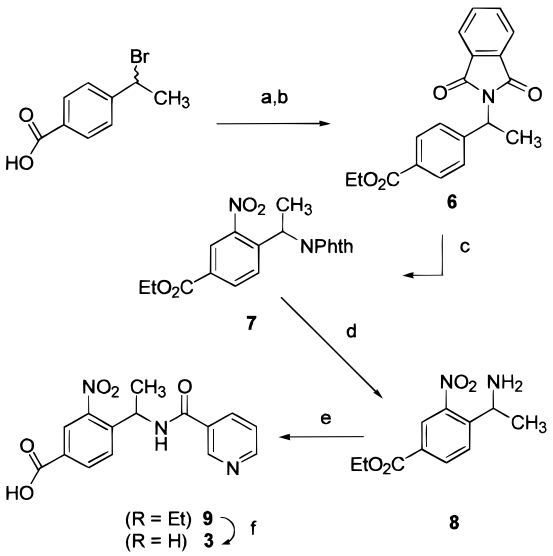
(15) Rich, D.; Gurawa, S. K. *J. Am. Chem. Soc.* **1975**, *97*, 1575.

(16) Toda, F.; Soda, Shinichi.; Goldberg, I. *J. Chem. Soc., Perkin Trans. 1* **1993**, *19*, 2357.

(17) No nitration of the phthalimide group was observed as elevated temperatures are typically required for nitration of such compounds. Nitration of **6** was accomplished in >95% yield as a mixture of diastereomers (5:1), compound **7**/2-nitro product.

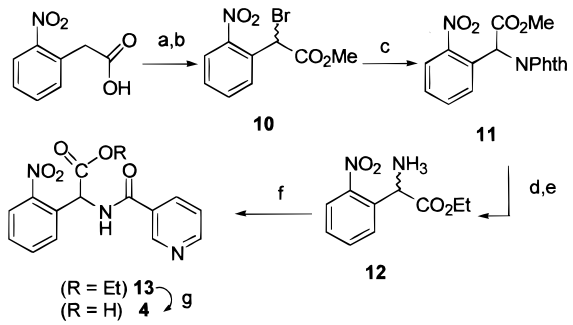
(18) Davis, A.; Smith, D. R.; Mcord, T. J. *J. Med. Chem*. **1973**, *16*, 1043.

**Scheme 1**[a]



[a] Reagents and conditions: (a) AcCl, EtOH, 0 °C to rt (92%); (b) $K^+$Phth, DMF, reflux (85%); (c) $HNO_3$, fuming, −10 °C (71%); (d) Ninhydrin, EtOH, 50 °C (80%); (e) DIEA, DMF, nicotinoylCl (91%); (f) $K_2CO_3$, MeOH/$H_2$ (87%).

**Scheme 2**[a]

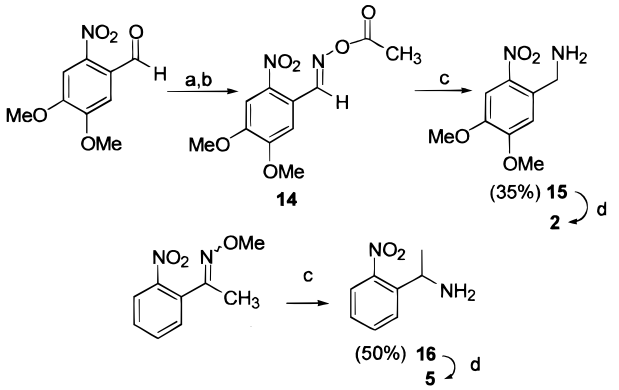


[a] Reagents and conditions: (a) MeI, DMF (95%); (b) NBS, $CCl_4$, reflux (22%); (c) $K^+$Phth, DMF (90%); (d) HCl/AcOH, reflux (80%); (e) *p*-TsOH, EtOH, reflux (88%); (f) nicotinoylCl, DMF, DIEA (82%), (g) $K_2CO_3$, MeOH/$H_2O$ (83%).

equiv of NBS in $CCl_4$ at reflux proceeded sluggishly over 3 days to provide **10** in 22% yield.[10b,20] The α-bromide was then carried forward following the procedure of Davis et al.[18] to furnish *o*-nitrophenyl glycine in high yield. Esterfication of *o*-nitrophenyl glycine then coupling with NCl afforded **13** while the free acid was generated as with **9** using $K_2CO_3$.

We have previously utilized $BH_3$·THF in the synthesis of *o*-nitrobenzylamine from *o*-nitrobenzaldehyde *O*-methyl oxime.[2] It was found that 6-nitroveratraldehyde *O*-methyl oxime provided only trace amounts of the desired amine using this method. Alternatively, we examined acetylated oxime **14** to enhance reduction via chelation of $BH_3$ with the acetyl group, Scheme 3.[14] Reduction of **14** with 6 equiv of $BH_3$·THF at reflux for 24 h resulted

**Scheme 3**[a]



[a] Reagents and conditions: (a) $NH_3OH$, pyridine/EtOH, reflux (89%); (b) $Ac_2O$, $Et_3N$, $CH_2Cl_2$ (73%), (c) $BH_3$/THF, reflux; (d) nicotinoylCl, DMF.

in amine **15** in 35% yield. The synthesis of amine **16** was completed by $BH_3$·THF reduction of *O*-methyl oxime-2-nitroacetophenone in 50% yield, Scheme 3. Coupling of **14** and **16** with NCl provided the desired caged nicotinamides **2** and **5**.

**Synthesis of Caged NADP Analogues.** The primary catalytic function of NADP glycohydrolases is the hydrolysis of NADP to ATPR (2′-monophosphoadenosine-5′-diphosphoribose) and nicotinamide.[21] Transglycosidase activity in the presence of modified pyridines and alcohols is found in mainly mammalian glycohydrolases such as sheep liver, porcine brain, and human cd3.[22] NADase has demonstrated a wide tolerance for altered pyridines but there are notable exceptions. In particular, carboxylic acid substituted pyridines have demonstrated a relatively low transglycosidation efficiency. Thus, the synthesis of NAADP (nicotinic acid adenine dinucleotide phosphate) from NADP using pig brain NADase requires a 20-fold greater concentration of nicotinic acid than analogues such as 3-acetylpyridine.[23a] Alternatively, ethyl nicotinate has been efficiently utilized during transglycosidation at only a modest excess followed by hydrolysis to form NAADP.[24] Hence, steric considerations or low water solubility should not be considered as the sole contributors to transglycosidation inefficiency.

NADase is commercially available (Sigma) as a water-insoluble acetone powder in which the enzyme is largely bound to microsomes. It is common practice that additional treatment is required to enhance both enzyme water solubility and activity. We have utilized a methodology derived from the preparations of Bernofsky and Kaplan to process the enzyme.[23] The synthesis of caged NADP **17** from nicotinamide **1**, NADP, and pig brain NADase was previously shown to achieve a modest yield of 20% using (3:1) **1**, NADP.[2] The general conditions used for the tranglycosidase reaction of nicotinamides (**1**−**5**) with NADP were carried out at 37 °C (pH = 7.3) for a reaction duration of 8 h. The enzyme was then separated by ultracentrifugation followed by $C_{18}$-HPLC purification. The enhanced hydrophobicity of the caged analogues conferred $C_{18}$ retention times substantially greater than

(19) α-Bromo-2-nitrophenyl acetic acid is available (5 mg/$75.0), Molecular Probes, Eugene OR.

(20) (a) Wieboldt, R.; Gee, K, R.; Niu, L.; Doraiswamy, R.; Carpenter, B. K.; Hess, G. P. *Proc. Nat. Acad. Sci. U.S.A.* **1994**, *91*, 8752.

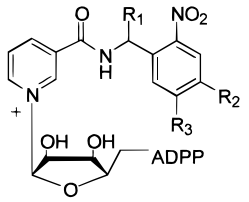
(21) (a) Woenckhaus, C.; Jeck, R. In *Pyridine Nucleotide Coenzymes: Chemical, Biochemical, and Medical Aspects, Part A*; Dolphin, D., Avramovic, O., Poulson, R., Eds.; Wiley: New York, 1987; pp 494−496. (b) Kaplan, N. O.; Ciotti, M, M.; Eys, J. V.; Burton, R. M. *J. Biol. Chem*. **1958**, *234*, 134.

(22) Berthelier, V.; Tixier, J, M.; Muller-Steffner, H.; Schuber, F.; Deterre, P. *Biochem. J*. **1998**, *330*, 1383.

(23) (a) Bernofsky, C. *Methods Enzymol.* **1980**, *66*, 105. (b) Colowick, S. P.; Kaplan, N. O. *Methods Enzymol.* **1955**, *2*, 660.

(24) Honjo, T.; Ikeda, M.; Andreoli, A.; Nishizuka, Y.; Hayaishi, O. *Biochim. Biophys. Acta* **1964**, *89*, 549.

those of ATPR or NADP, thus facilitating the separation of large excesses of these two compounds.




**17** ($R_1$= H, $R_2$ = H, $R_3$ = H)
**18** ($R_1$= H, $R_2$ = OMe, $R_3$ = OMe)
**19** ($R_1$ = $CO_2$, $R_2$ = H, $R_3$ = H)
**20** ($R_1$ = $CH_3$, $R_2$ = $CO_2H$, $R_3$= H)
**21** ($R_1$ = $CH_3$, $R_2$ = H, $R_3$ = H)

Caged nicotinamide **2** was used at only a 1.2-fold excess (relative to NADP) due to its marginal water solubility but provided a modest yield of 9% caged NADP **18**. Synthesis of both **19** and **20** required dramatically higher concentrations of their respective nicotinamides to achieve practical yields. Thus, the concentrations of **4** and **3** were increased to ~0.1 M (sodium salt), a 20-fold excess of nicotinamide analogue to NADP.[23a] Following enzyme removal, remaining **3** and **4** were largely recovered via precipitation with 3 N HCl at 4 °C. The filtrate was then purified to provide **19** (9% yield) and **20** (7% yield).

It was early in our studies that we attempted the synthesis of caged NADP analogue **21**. The low water solubility of **5** (~1.0 mM, 37 °C) was an anticipated difficulty for aqueous transglycosidation. Reaction of **5** with NADase resulted in only a trace yield of (as determined by HPLC, UV) analogue **21**. Addition of Igepal CO520 surfactant, to solubilize large excesses of nicotinamide analogue, was proven advantageous in the NADase-catalyzed synthesis of thio-NADP.[25] Repetition under the prior conditions with 20% Igepal CO520 and **5** (~3 mM), failed to enhance the product yield.

**Optical Investigation and Enzyme Activation.** The phototriggering of enzyme activation can best be assessed through a course of study that considers both optical changes and detailed product analysis. We investigated optical changes (UV, fluorescence) accompanying UV irradiation of compounds **18**, **19**, and **20** so as to establish parameters for background, optical "noise" calibration. A simple assay procedure was used in which **18**, **19**, and **20** were progressively photolyzed while UV and fluorescence changes monitored. Following photolysis completion, alcohol dehydrogenase (AD) and 2-propanol were added in order to calculate NADP release based upon NADPH formation (340 nm detection). The potential reaction of NADPH with nitroso-photobyproducts was a major concern as these would likely be translated into misleading optical signals. Dithioerythritol (DTE) was therefore present to serve as a scavenger of the expected *o*-nitroso byproducts, while basic conditions (pH = 7.9) that optimized both dehydrogenase activity and NADPH stability were chosen.[26] The use of DTE has proven effective in scavenging nitroso byproducts since its first use by Kaplan et al.[12]

Figure 3a−c demonstrates the UV−vis spectral changes that occurred commensurate with UV irradiation. Photolysis of compound **18** resulted in a modest red shift (350−375 nm) of the secondary absorption maxima, while photolysis of **20** exhibited only a minor increase in the UV range. A substantial rise in absorption between 300 and 500 nm occurred upon irradiation of compound **19**, resulting in an intense yellow solution color ($\lambda_{max}$ = 400 nm), Figure 3f. This optical change is strikingly similar to the new absorption noted by Hess et al. following the photolysis of *N*-(α-carboxy-2-nitrobenzyl)carbamoylcholine.[27] The addition of AD to the post photolysis solutions of **18**, **19**, and **20** provided the anticipated increase in absorption at 340 nm resulting from NADPH formation. Yields of 27%, 35%, and 71% NADP released were derived from the respective absorption changes. The absence of substantial UV spectral variation and apparently high net photorelease demonstrated by **20** allowed for the unobscured observation of dihydropyridine formation in the presence of AD. Hence, UV irradiation of **20**, 2-propanol, and AD were carried out under the solution conditions used for the UV−vis studies (Figure 3a−c). Figure 3d demonstrates a progressive generation of NADPH by AD that was unaffected by either modest UV exposure or the presence of the released *o*-nitrosoketone (or DTE adducts).

The prominent fluorescence of NAD(P)H at ~465 nm upon 340 nm excitation has been widely utilized in applications that range from pure biology and kinetics to commercial biosensors.[28] It is noteworthy that none of the caged compounds displayed fluorescence activity prior to UV exposure. The fluorescence changes of compounds **18**, **19**, and **20** following complete photolysis were studied under the same solution conditions used in the previous UV−vis studies, Figure 3e. The fluorescence spectrum of photolyzed **18** produced no substantial change from background, while irradiated **20** generated only a minor fluorescence contribution at 465 nm. Upon addition of AD and 2-propanol, the post photolysis solution of **20** displayed an unambiguous NADPH fluorescence profile. Complete photolysis of compound **19** resulted in the generation of an intensely fluorescent element with an emission maximum at 409 nm. The substantial fluorescence emission contribution at ~465 nm prevented the clear-cut differentiation of NADPH from background.

**NADP Photorelease:HPLC/MS Analysis.** The relevance of biological stimulation induced by the photolysis of caged compounds is as much a function of the released byproduct(s) as it is the desired product. Photostimulation and photoinactivation of enzyme activity may go hand in hand. There have been few studies in the literature of *o*-nitrobenzyl-caged compounds that confront this issue despite the fact that the released *o*-nitroso byproducts are notorious for adduct formation.[12] The efficient scavenging by dithiothreitol (DTT) of the *o*-nitrosoketone byproduct released by **20** has been thoroughly investigated by Trentham et al.[11a]

An HPLC study, Figure 4, of the photolysis behavior of **18**, **19**, and **20** was conducted so as to confirm the spectral variations attributed to both NADPH and back-

(25) Nakanishi, K.; Mitsushima, H.; Baba, Y. Japan. 7214, 919 (Cl.C.12d,C07d), May 4, 1972, Applications 6922.552, March 25, 1969.

(26) NAD(P) achieve maximum stability at acidic pH while NAD(P)H are most stable under basic conditions. NAD(P)H decomposition follows hydration of the dihydropyridine group under acidic conditions.

(27) Milburn, T.; Matsubara, N.; Billington, A. P.; Udgaonkar, J. B.; Walker, J. W.; Carpenter, B. K.; Webb, W. W.; Marque, J.; Denk, W.; McCray, J. A.; Hess, G. P. *Biochemistry* **1989**, *28*, 49.

(28) (a) *Handbook of Biosensors and Electronic Noses: Medicine, Food and the Environment*; Erika, K. R., Ed.; CRC Press: New York, 1995. (b) Yamanaka, S, A.; Dunn, B.; Valentine, J. S.; Zink, J. I. *J. Am. Chem. Soc.* **1995**, *117*, 9095.

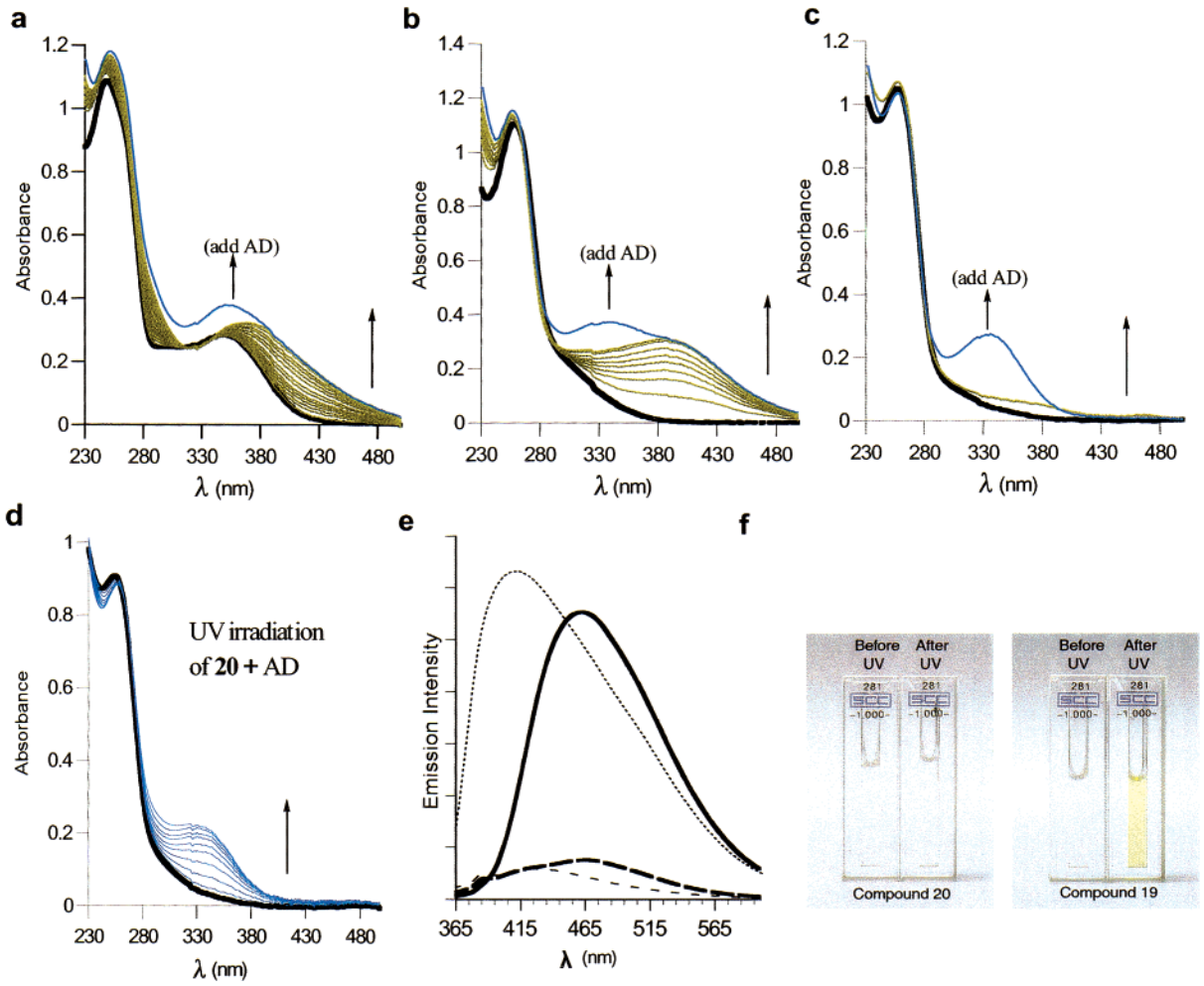



**Figure 3.** Changes in UV−vis, fluorescence spectra of compounds **18**, **19**, and **20** (∼0.05 mM caged analogue, pH = 7.9, 50 mM Trizma, 0.45 mM DTE) upon UV exposure (0−450 s). Panels a−c: UV−vis spectral changes followed by addition of alcohol dehydrogenase (AD), 2-propanol; (d) photolysis of **20** (0.041 mM) in the presence of AD and 2-propanol (0.5 mM); (e) fluorescence emission spectra (340 nm excitation); prephotolysis of **18**, **19**, and **20** (- - -); post-photolysis of **20** (— —); post-photolysis mixture **20** with AD and 2-propanol (solid); post-photolysis of **19**, no AD (···); (f) photograph of pre- and postphotolysis of **19** and **20** under stated conditions.

ground in Figure 3. Given the possibility that enzymatically generated NADPH might undergo oxidative or alkylative side reactions with byproducts, distortions in optically derived yields would be expected. Figure 4a−c shows HPLC traces corresponding to the photolysis of compounds **18**, **19**, and **20**, respectively. Figure 4d provides progress plots for the appearance of NADP over the course of photolysis of compounds **18**, **19**, and **20**. Analogues **18** and **19** conformed closely with their optically derived values and demonstrated 22% and 25% NADP photoreleased. The net yield of NADP from α-methyl-*o*-nitrobenzyl analogue **20** was measured at 75%, a close match with the enzymatically measured value. The post-photolysis mixture of **20** was shown to be relatively clean with only several minor byproducts. In additional experiments (similar pH, etc.), it was found that increasing the concentration of DTE 20-fold to 8 mM prior to photolysis of **18**, **19**, and **20** produced no increase in NADP release.

Remarkably, the HPLC traces of compounds **18** and **19** each demonstrated the formation of a single primary byproduct peak.[29] The UV spectra of the collected byproduct peaks associate them with the major spectral changes arising upon photolysis of **18** and **19**, Figure 4a,b.[30] The compound **19** byproduct was also found to be the source of the fluorescence activity displayed by **19** following UV exposure, Figure 3e. The high condition sensitivity of *o*-nitrobenzyl photochemistry prompted the further study of these byproducts by ESI/MALDI mass spectroscopies. Initially, it was thought that the nitrosoaldehyde, nitrosopyruvate products had reacted with N-1 or N-6 of adenine. This would be consistent with the tendency of these groups to undergo alkylation[31] and possibly be predisposed by the base-stacked conformer of NADP.[32] We were surprised to find the mass spectra of both

(29) Overexposure of these byproducts with UV or 25 °C storage has been shown to result in sample decomposition. After MS sample submission, samples were kept at −20 °C until mass analysis. Storage at −20 °C provides sample preservation for several weeks without decomposition.

(30) See the Supporting Information for UV spectra of the byproducts.

(31) (a) Alvarez, K.; Vasseur, J.-J.; Beltran, T.; Imbach, J.-L. *J. Org. Chem.* **1999**, *64*, 6319. (b) Zapelli, P.; Rossodivita, A.; Re, L. *Eur. J. Biochem.* **1975**, *54*, 475. (c) Windmuller, H. G.; Kaplan, N. O. *J. Biol. Chem.* **1961**, *236*, 2716.

(32) NAD(P) has been shown to exist at 25 °C in one of four possible base-stacked conformers (∼50% population) involving the nicotinamide and adenine groups. This state is in rapid equilibrium with an extended conformer.

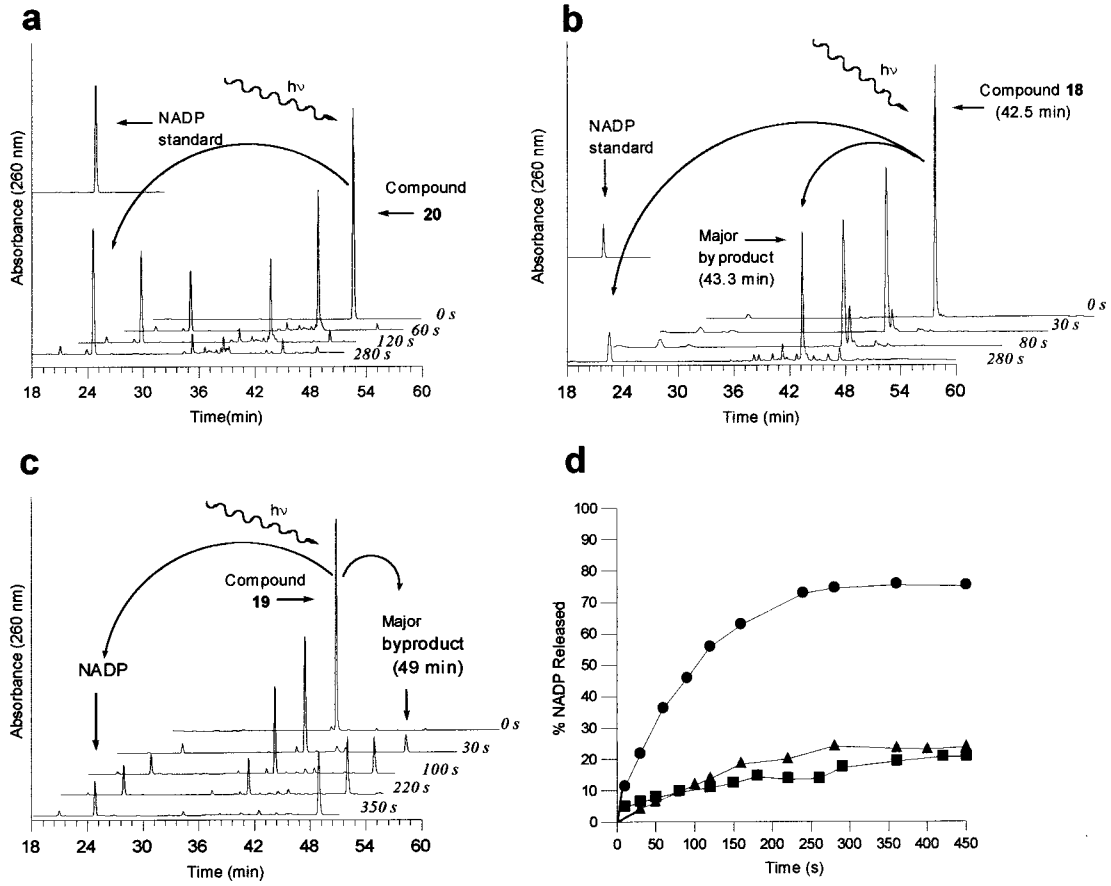



**Figure 4.** Photolysis of compounds **18**, **19**, and **20** (panels a−c). Conditions: **18**, **19**, and **20** (~0.05 mM caged analogue, pH = 7.3, 0.45 mM DTE, 50 mM Trizma; (d) progress plots for photolysis of compounds **18**, **19**, and **20**; ■ (compound **18**); ▲ (compound **19**); ● (compound **20**). See the Experimental Section for further details.

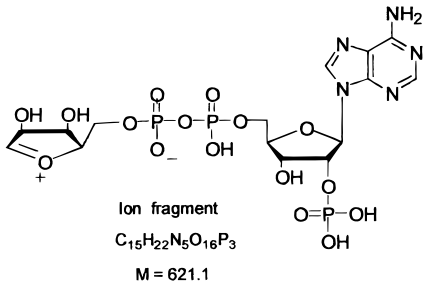
**Table 1. Primary Ion Fragments for NADP, 19, and Byproducts**[a]

| compd | ESI(+) (*m*/*z*) | ESI(−) (*m*/*z*) | MALDI (*m*/*z*) |
|---|---|---|---|
| NADP | 622 | 620 | 622 |
| **19** | 622 | 620 | 622 |
| **18** byproduct | 622 | 620 | |
| **19** byproduct | 622 | 620 | 622 |

[a] See the Supporting Information for complete mass spectra. Primary ion fragment M = 621.1. ESI(+) and MALDI peaks represent $[M + H]^+$. ESI(−) corresponds to $[M - H]^-$.

byproducts supported an NADP adduct of nicotinamide and the photolyzed nitroso compound.

The first primary ion fragment of NADP or caged NADP is typically the loss of nicotinamide (or caged nicotinamide) to produce the ADPP oxonium 621 *m*/*z* ion fragment.[33] This mass peak is often considerably more intense than the molecular ion peak and is considered a characteristic sign of NAD(P). The mass spectra of the major byproducts of compounds **18** and **19** each showed the *m*/*z* 621 ion fragment as a dominant peak, thus signaling the preservation of the ADPP substructure, Table 1.[34] It was a manifestation of the highly labile nature of these nicotinamide adducts that we were unable to confidently assign a molecular ion mass for these byproducts.



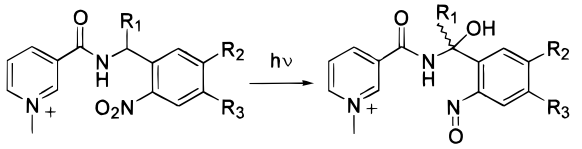

The chemical significance of nicotinamide adduct formation reflects rapid recombination or rearrangement following photolysis. It explains our inability to enhance NADP yields by increasing DTE concentrations. The biological relevance of nicotinamide versus adenine adducts gains significance from each products distinctive influence on the well-established ADPP enzyme binding domain of NADP. Due to their uncompromised ADPP substructure, nicotinamide modified adducts would be relatively unhindered competitors for enzyme binding sites while adenine alkylation would hamper tight enzyme-byproduct association.

Since the seminal work of Kaplan et al.,[12] only the release of ATP has been rigorously demonstrated to occur under aqueous conditions in a synchronous fashion with the photoinduced cleavage of an α-methyl-*o*-nitrobenzyl group from the γ-phosphate.[3,35] This precise mechanism has not been proven to encompass alternative leaving groups. It has been proposed that the *aci*-nitro excited state of *o*-nitrobenzyl-protected carboxamides decays to

(33) Schulten, H. R.; Schiebel, H. M. In *Pyridine Nucleotide Coenzymes: Chemical, Biochemical, and Medical Aspects, Part A*; Dolphin, D., Avramovic, O., Poulson, R., Eds.; Wiley: New York, 1987; pp 232−250.

(34) See the Supporting Information for ESI, MALDI mass spectral analysis of NADP, **19**, isolated byproducts.

an *N*-(α-hydroxybenzyl)amide (NHA) intermediate of indeterminate lifetime.[3d,8] Peysner showed that photolysis of *N*-(2-nitrobenzyl)-1-naphthamide at −70 °C produced a nitroso compound, (*N*-α-hydroxy-2-nitrosobenzyl)-1-naphthamide, as the major product.[36] The synthesis of α-hydroxy(glycine) peptides by Steglich[37] and *N*-(α-hydroxybenzyl)benzamide derivatives by Katitzky et al.[38] testify to the potential stability of an NHA precursor. Such intermediates may tether nitrosobenzyl compounds for extended periods prior to substrate release, thus enhancing the likelihood for further inter- and intramolecular chemistry.




*N*-(α-hydroxybenzyl)amide Intermediate

As far as intramolecular adduct formation is concerned, the hydrolysis of the NHA intermediate to the corresponding imine is one possibility.[39] Only primary and secondary amines are known to react with nitrosoaromatic compounds to form diazo compounds,[40] although the potential reactivity of amides with nitrosoaromatics is still largely unstudied. In the event of a prolonged NHA lifetime the possibility that NHA photoreactivity may itself compete with product release is worthy of consideration. With regard to a presumed NHA state for **19**, mandelic acid[41] has been shown to undergo highly facile heterolytic photodecarboxylation (quantum efficiency product formation = 0.4) under aqueous conditions. The resulting α-benzyl carbanion would likely be delocalized into the adjacent aromatic group. Indeed, the new UV absorption at 400 nm possessed by **19** is very similar to the absorption change observed by Wan[42] to accompany carbanion delocalization following UV-induced aqueous decarboxylation of *p*-nitrophenylalanine. The ∼10 min increase in the $C_{18}$ HPLC retention time for the byproduct of **19** (relative to the parent compound) is indicative of the loss of hydrophilicity that would accompany decarboxylation. Furthermore, the efficient radical-based photodecarboxylation of similar structural arrangements has also been demonstrated by the pivaloylglycol photolabile linker.[43] Whether the high net yield of photorelease demonstrated by **20** is reflective of an attenuated NHA lifetime will require further study.

## Conclusions

We have demonstrated the successful enzymatic synthesis of three caged NADP analogues with differing photochemistries. The incorporated caged nicotinamides represent some of the most sterically hindered groups ever exchanged via direct enzymatic transglycosidation. Substituents at the α-benzyl position of caged nicotinamides have been shown to contribute substantially to transglycosidation inefficiency. The net photoconversions highlight the differing photorelease properties of α-benzyl substituents and establish the α-methyl-*o*-nitrobenzyl group as providing optimal aqueous release of NADP within the range of compounds **17**−**20**. The poor release yields demonstrated by an α-carboxy-*o*-nitrobenzyl group indicates that generic α-susbstitution is not necessarily a panacea for low release yields. The formation of photobyproducts has been shown to be a substantial drawback for certain caged NADP analogues.[44] These results highlight the need for rigorous chromatographic analysis when assessing caged compound performance.

## Experimental Section

**General Methods.** Silica chromatography utilized Fisher (200−425) mesh gel, and thin-layer chromatography was performed on Whatman (250 μM, 60 Å) plates. THF was distilled over Na/benzophenone. Dry DMF and *N*,*N*-diisopropylethylamine (99.5%) (DIEA) were purchased from Aldrich. Mass spectra were carried out at the The Scripps Research Institute, La Jolla, CA.

**General Procedures for Photolysis of 18, 19, and 20.** All photolyses were performed using a Bausch and Laumb 150 W xenon arc lamp with <300 nm cutoff filter. Aqueous solutions for each timepoint used in HPLC analysis consisted of **18**, **19**, and **20** (∼0.05 mM), 1 mL, 50 mM Trizma, pH = 7.3, 0.4 mM dithioerythritol (DTE). Only fresh DTE (99%, Sigma) was used (−20 °C storage under argon). Solutions were placed in a 1 cm quartz cuvette with stir bar and sealed. HPLC conditions are as described for purity analysis of compound **20**. UV−vis spectra for **18**, **19**, and **20** (∼0.05 mM) photolysis were performed on a Perkin-Elmer Lambda 3B UV−vis spectrophotometer. Each series of UV−vis spectra for **18**, **19**, and **20** were obtained from a single 1 mL sample (above solution conditions, cuvette, except pH = 7.9) followed by the addition of alcohol dehydrogenase (Thermoanerobium brockii, Sigma), 2.1 units (2 μL), 2-propanol (10 μL, 0.16 M). The small UV contribution (230−300 nm) of the enzyme was subtracted from the final spectrum. Fluorescence emission spectra were obtained from a Fluoro-max2 fluorimeter using 340 nm excitation. Sample UV irradiations for fluorescent studies were performed on 2 mL sample volumes using the solution conditions described for UV−vis studies. The fluorescence emission spectrum of alcohol dehydrogenase at 340 nm excitation was similar to background.

***N*-(4,5-Dimethoxy-2-nitrobenzyl)nicotinamide (2).** Amine **15** (1.0 g, 4.69 mmol) was added to dry DMF (40 mL). Nicotinoyl chloride HCl (1.24 g, 7.0 mmol) (Aldrich) was then added followed by DIEA (3.7 mL, 21 mmol). The solution was stirred for 3 h, after which time the DMF was removed under reduced pressure. The remaining solid was suspended in $CH_2Cl_2$ (300 mL) and extracted with saturated sodium bicarbonate and then $H_2O$. The organic layer was dried with $MgSO_4$ followed by filtration. The solvent was removed under reduced pressure and the remaining solid purified by silica flash

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(36) Peysner, J. R.; Flechtner. *J. Org. Chem*. **1987**, *52*, 4646.

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(38) Katritzky, A, R.; Rao, M.; Suresh, C. *Synthesis* **1990**, *8*, 663.

(39) (a) Bundgaard, H.; Johansen, M. *Int. J. Pharm*. **1984**, *22*, 45. (b) Sayer, J. M.; Conlon, P. *J. Am. Chem. Soc.* **1980**, *102*, 3592. (c) Reference 39a provides useful insights into the kinetics of imine formation from NHA-like compounds.

(40) Zuman, P.; Shah, B. *Chem. Rev.* **1994**, *22*, 1621.

(41) (a) Wan, P.; Xu, X. *Tetrahedron Lett*. **1990**, *31*, 2809. (b) Wan, P.; Budac, D. In *CRC Handbook of Organic Photochemistry and Photobiology*; Horspool, W. H., Song, P.-S., Eds.; CRC press: London, 1995; pp 384−389.

(42) Wan, P.; Lee, C. K. J. *Photochem. Photobiol. A* **1993**, *76*, 39.

(43) Peukert, S.; Giese, B. *J. Org. Chem*. **1998**, *63*, 9045.

(44) (a) This work is largely in disagreement with both the synthetic conclusions and solution photochemistry performed on compound **19** by Cohen et al. (ref 44b). The absence of UV−vis studies or HPLC progress plots makes their work difficult to interpret in light of the highly ambiguous nature of *aci*-nitro decay from *o*-nitrobenzyl modified carboxamides. (b) Cohen, B. E.; Stoddard, B. L.; Koshland, D. E. *Biochemistry* **1997**, *36*, 9035.

chromatography (10% MeOH, $CH_2Cl_2$, 0.5% DIEA) to give 0.94 g of **2** as a yellow solid, 63% yield. $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ 3.93 (s, 3 H), 4.0 (s, 3 H), 4.82 (2, 1 H), 4.84 (s, 1 H), 7.17 (s, 1 H), 7.34−7.38 (2 H), 7.66 (s, 1 H), 8.06 (dt, $J$ = 2 Hz, $J$ = 8 Hz, 1 H), 8.70 (s, 1 H), 8.99 (s, 1 H). $^{13}$C NMR ($CDCl_3$, 400 MHz): $\delta$ 42.2, 56.4, 56.7, 108.0, 114.4, 123.3, 128.0, 134.7, 138.0, 140.5, 147.9, 148.3, 152.2, 153.5, 165.3. HRMS *m*/*z* calcd for $C_{15}H_{15}N_3O_5$ (M + 1) 318.1090, found 318.1080.

***N*-(Ethyl 4-(1-amino)ethyl-3-nitrobenzoate)nicotinamide (3).** Compound **9** (4.0 g, 11.7 mmol) was placed in MeOH (150 mL) followed by the addition of $H_2O$ (38 mL). The solution was cooled to 4 °C, and $K_2CO_3$ (3.23 g, 23.4 mmol) was added over 10 min. The solution was then stirred for 20 h. The MeOH was removed under reduced pressure, and enough $H_2O$ was added to bring the remaining solution volume to 60 mL. This solution was then extracted with $CH_2Cl_2$. The aqueous layer was saved and the solution acidified to pH = 2.0 with concentrated HCl. The remaining solvent was removed under reduced pressure. Concentrated HCl, 10% in 1,4-dioxane (30 mL), was added and the solvent removed under reduced pressure to result in a white powder. The powder was thouroughly mixed in $H_2O$ (4.0 mL) followed by filtration. Drying for 48 h in vacuo resulted in 3.6 g of nicotinamide **3** in 87% yield, HCl salt. $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 1.30 (t, $J$ = 6.9 Hz, 3 H), 1.59 (d, $J$ = 6.9 Hz, 3 H), 4.33 (q, $J$ = 7.2 Hz, 2 H), 5.45 (m, 1 H), 7.49 (m, 1 H), 7.91 (d, $J$ = 8.1 Hz, 1 H), 8.15−8.25 (2 H), 8.34 (d, $J$ = 1.5 Hz, 1 H), 8.69 (s, 1 H), 9.00 (s, 1 H), 9.36 (d, $J$ = 6.3 Hz, 1 H). $^{13}$C NMR (DMSO-$d_6$, 400 MHz): $\delta$ 45.8, 55.6, 116.6, 118.5, 121.1, 122.2, 122.8, 124.5, 125.3, 134.2, 135.5, 136.4, 140.1, 154.6, 161.7. HRMS: *m*/*z* calcd for $C_{15}H_{13}N_3O_5$ (M + 1) 316.0933, found 316.0942.

***N*-(Ethyl 2-nitrophenylglycine)nicotinamide (4).** Compound **13** (1.7 g, 6.54 mmol) was saponified over 10 h using the method described for (**3**). Following purification, the remaining solid was dried in vacuo for 48 h to give 1.78 g of **4** as a white powder in 83% yield. $^1$H NMR ($DOCD_3$, 400 MHz): $\delta$ 6.41 (s, 1H), 7.56 (m, 1 H), 7.65−7.69 (2 H), 7.94 (m, 1 H), 8.03 (d, $J$ = 8.8 Hz, 1 H), 8.72 (dt, $J$ = 1.2 Hz, 6.8 Hz, 1 H), 8.85 (s, 1 H), 9.14 (s, 1 H).$^{13}$C NMR ($DOCD_3$, 400 MHz) 55.3, 126.0, 128.0, 130.6, 131.6, 132.3, 134.0, 134.7, 143.6, 144.9, 145.9, 149.6, 164.1, 172.2. HRMS: *m*/*z* calcd for $C_{14}H_{12}N_3O_5$ (M − H + Na) 324.0596, found 324.0583.

***N*-1-(2-Nitrophenyl)ethylnicotinamide (5).** Compound **16** (1.04 g, 6.23 mmol) was coupled with nicotinoyl chloride and purified using the method described for compound **2**. Following purification, **5** was afforded in 1.6 g as a pale yellow solid, 90% yield. $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$ 1.59 (d, $J$ = 6.9 Hz, 3 H), 5.62 (m, 1 H), 7.27−7.34 (2 H), 7.50 (t, $J$ = 8.1, 1 H), 7.56−7.58 (1 H), 7.73 (d, $J$ = 6.9 Hz, 1 H), 7.79 (d, $J$ = 7.8 Hz, 1 H), 8.06 (d, $J$ = 8.1 Hz), 9.04 (s, 1 H). $^{13}$C NMR ($CDCl_3$, 300 MHz): $\delta$ 21.3, 46.9, 123.6, 124.6, 128.0, 128.3, 133.4, 129.8, 135.8, 138.3, 147.4, 148.4, 151.2, 164.4. HRMS: *m*/*z* calcd for $C_{14}H_{13}N_3O_3$ (M + 1) = 272.1035, found 272.1027.

**Ethyl 4-(1-Phthalimido)ethylbenzoate (6).** (a) 4-(1-Bromoethyl)benzoic acid (Lancaster) (10 g, 43.8 mmol) was added to anhydrous EtOH (100 mL). The solution was placed under argon and cooled to −10 °C. AcCl (12.4 mL, 175 mmol) was then slowly added via syringe. The solution was brought to 0 °C and then stirred for 0.5 h followed by 25 °C, 24 h. Crushed ice, 600 g, was then added. After 5 min, saturated sodium bicarbonate (250 mL) was slowly added. To the aqueous slurry was added $CH_2Cl_2$ (600 mL) and the solution extracted. The organic phase was saved and further extracted with 20% sodium bicarbonate and $H_2O$. The solution was then dried with $MgSO_4$ followed by filtration. The $CH_2Cl_2$ was removed under reduced pressure to yield 10.3 g of ethyl 4-(1-bromoethyl)-benzoic acid as a yellow liquid in 92% yield. $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ 1.37 (t, $J$ = 7.2 Hz, 3 H), 2.02 (d, $J$ = 6.8, 3 H), 4.36 (q, $J$ = 7.2 Hz, 2 H), 5.18 (q, $J$ = 6.8 Hz, 1 H), 7.48 (d, $J$ = 8.4 Hz, 2 H), 8.00 (d, $J$ = 8.4 Hz, 2 H). $^{13}$C NMR ($CDCl_3$, 400 MHz): $\delta$ 14.4, 26.6, 48.0, 61.0, 126.6, 129.6, 129.7, 147.6, 165.7. HRMS: *m*/*z* calcd for $C_{11}H_{13}O_2Br$ (M + 1) 259, found 259.

(b) Ethyl-4-(1-bromoethyl)benzoic acid (9.4 g, 36.4 mmol) was added to dry DMF (30 mL) followed by potassium phthalimide (6.78 g, 36.6 mmol) and the mixture refluxed under argon for 1 h. The DMF solution was then added directly to 550 mL $CH_2Cl_2$ and extracted with 10% saturated sodium bicarbonate then $H_2O$. The solvent was removed under reduced pressure to result in 10 g of **6** as a waxy solid, 85% yield. $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$ 1.36 (t, $J$ = 7.2 Hz, 3 H), 1.93 (d, $J$ = 7.5 Hz, 3 H), 4.35 (q, $J$ = 7.2 Hz, 2 H), 5.60 (q, $J$ = 7.5 Hz, 1 H), 7.55 (d, $J$ = 8.1 Hz, 2 H), 7.68−7.71 (2 H), 7.80−7.83 (2 H), 8.00 (d, $J$ = 8.4 Hz, 2 H). $^{13}$C NMR ($CDCl_3$, 300 MHz): 14.0, 17.5, 49.2, 60.1, 123.2, 127.2, 129.6, 129.7, 131.7, 133.9, 144.9, 166.1, 167.8. HRMS: *m*/*z* calcd for $C_{19}H_{17}NO_4$ (M + 1) 324.1236, found 324.1230.

**Ethyl 3-Nitro-4-(1-phthalimido)ethylbenzoate (7).** Compound **6** (9.7 g, 30 mmol) was placed in a 250 mL round-bottom flask and cooled to −10 °C. Fuming nitric acid (100 mL) was cooled to −10 °C and added to the flask. The reaction was stirred at −10 °C for 1 h, after which time the solution was poured onto ~600 g of crushed ice. The slurry was poured onto a large fritted funnel (40 Å) and the product slowly accumulated as a white precipitate until all the ice had melted. The remaining white solid was then washed with $H_2O$ (600 mL). The wet solid was then dissolved and washed through the funnel with 500 mL of $CH_2Cl_2$. The $CH_2Cl_2$ was then extracted with $H_2O$, 10% sodium bicarbonate, and then $H_2O$. The $CH_2Cl_2$ was dried with $MgSO_4$ and filtered. The remaining solid was purified by silica flash chromatography (30% ethyl acetate, hexanes, 0.5% DIEA) to give 7.8 g of **7** as a pale yellow solid, 71% yield. $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ 1.39 (t, $J$ = 7.2 Hz, 3 H), 1.98 (d, $J$ = 7.2 Hz, 3 H), 4.40 (q, $J$ = 7.2 Hz, 2 H), 6.08 (q, $J$ = 7.2 Hz, 1 H), 7.70−7.27 (2 H), 7.80−7.82 (2 H), 7.99 (d, $J$ = 8.4 Hz, 1 H), 8.24 (dd, $J$ = 1.6 Hz, 8.2 Hz, 1 H), 8.44 (s, $J$ = 2 Hz, 1H). $^{13}$C NMR ($CDCl_3$, 400 MHz): $\delta$ 14.3, 18.3, 45.8, 61.8, 123.4, 125.3, 129.9, 131.1, 131.4, 131.6, 133.3, 134.2, 139.1, 169.9, 167.6. HRMS: *m*/*z* calcd for $C_{19}H_{16}N_2O_6$ (M + 1) 360.1087, found 369.1099.

**Ethyl 4-(1-Amino)ethyl-3-nitrobenzoate (8).** Compound **7** (7.5 g, 20.3 mmol) was added to anhydrous EtOH (80 mL). Hydrazine−$H_2O$, 85% w/v (1.34 mL, 21.3 mmol), was added and the solution heated to 50 °C for 1.5 h. The solution was then cooled to 25 °C, and hydrazine−$H_2O$, 85% w/v (1.34 mL, 21.3 mmol), was added. The solution was again heated to 50 °C for an additional 45 min. The resulting precipitate was then filtered to separate the phthalyl hydrazide salt. The filtrate was saved and the EtOH removed under reduced pressure. $CH_2Cl_2$ (250 mL) was added to the remaining oil followed by extraction with $H_2O$. The $CH_2Cl_2$ was then dried with $MgSO_4$ followed by filtration. The solvent was removed under vacuum followed by silica chromatography (5% MeOH, $CH_2Cl_2$, 0.5% DIEA). The solvent was then removed under reduced pressure followed by addition of 10% concentrated HCl−1,4 dioxane (30 mL). The dioxane was then removed under reduced pressure to result in 4.44 g of amine **8** as the HCl salt, 80% yield. NMR characterization carried out on the free amine. $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ 1.39 (t, $J$ = 7.2 Hz, 3 H), 1.44 (d, $J$ = 6.3 Hz, 3 H), 4.62 (q, $J$ = 6.6 Hz, 1 H), 7.89 (d, $J$ = 8.1 Hz, 1 H), 8.22 (dd, $J$ = 1.5 Hz, 8.1 Hz, 1 H), 8.38 (d, $J$ = 1.8 Hz, 1 H). $^{13}$C NMR ($CDCl_3$, 400 MHz): $\delta$ 14.1, 24.6, 46.0, 61.5, 124.7, 127.6, 129.6, 133.1, 146.2, 148.3, 163.9. HRMS: *m*/*z* calcd for $C_{11}H_{15}N_2O_4$ (M + 1) 239.1032, found 239.1037.

***N*-(Ethyl 4-(1-amino)ethyl-3-nitrobenzoate)nicotinamide (9).** Compound **8** (4.0 g, 14.5 mmol) was coupled with nicotinoyl chloride and purified using the method described for compound **2**. Purification provided **9** (4.5 g) as a light yellow oil, 91% yield. $^1$H NMR (DMSO-$d_6$, 300 MHz): $\delta$ 1.30 (t, $J$ = 6.9 Hz, 3 H), 1.59 (d, $J$ = 6.9 Hz, 3 H), 4.33 (q, $J$ = 7.2, 2 H), 5.45 (m, 1 H), 7.49 (m, 1 H), 7.91 (d, $J$ = 8.1 Hz, 1 H), 8.15−8.24 (2 H), 8.34 (d, $J$ = 1.5 Hz, 1 H), 8.69 (s, 1 H), 9.00 (s, 1 H), 9.36 (d, $J$ = 6.3 Hz, 1 H). $^{13}$C NMR (DMSO-$d_6$, 300 MHz): $\delta$ 14.1, 20.9, 45.1, 61.4, 123.2, 124.2, 128.4, 129.0, 129.4, 133.4, 134.9, 144.3, 148.0, 148.2, 151.8, 163.5, 164.4. HRMS: *m*/*z* calcd for $C_{17}H_{17}N_3O_5$ (M + 1) 344.1246, found 344.1256.

**Methyl α-Bromo-2-nitrophenylacetate (10).** (a) *O*-Nitrophenylacetic acid (20 g, 109 mmol) (Aldrich) was dissolved in dry DMF (350 mL) along with sodium bicarbonate (18.5 g, 175 mmol). The solution was allowed to stir for 15 min, after

which methyl iodide (77 g, 550 mmol) was added. The reaction was allowed to proceed for 8 h under argon. The solvent was then removed under reduced pressure, using suitable solvent traps. The resulting yellow liquid was added to $CH_2Cl_2$ (700 mL) and extracted with $H_2O$ and then 10% sodium bicarbonate. The organic layer was then dried with $MgSO_4$ and filtered and the solvent removed under reduced pressure to result in 20.4 g of methyl 2-nitrophenylacetate as a yellow oil in 95% yield. $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ 3.76 (s, 3 H), 4.09 (s, 2 H), 7.42 (d, $J$ = 7.6 Hz, 1 H), 7.53 (t, $J$ = 7.6 Hz, 1 H), 7.66 (t, $J$ = 7.2 Hz, 1 H), 8.43 (d, $J$ = 8 Hz, 1 H). $^{13}$C NMR ($CDCl_3$, 400 MHz): $\delta$ 42.4, 53.7, 124.8, 129.8, 130.4, 132.9, 133.7, 147.3, 167.8. MS: $m/z$ calcd for $C_9H_9N_1O_4$ (M + 1) 196, found 196.

(b) Methyl 2-nitrophenylacetate (17.9 g, 92 mmol) was dissolved in $CCl_4$ (350 mL) along with benzoyl peroxide (0.353 g, 1.46 mmol). The solution was refluxed under argon for 24 h. The reaction was then cooled and the precipitated succinamide filtered. NBS (19.6 g, 110 mmol) and benzoyl peroxide (0.353 g) were again added, and the solution was refluxed under argon for an additional 48 h. The oil was purified directly on a large silica column (2% MeOH, $CH_2Cl_2$) to result in 5.5 g bromide **10** as a red oil, 22% yield. $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ 3.81 (s, 1 H), 6.08 (s, 1 H), 7.53 (t, $J$ = 7.6 Hz, 1 H), 7.70 (t, $J$ = 6.8 Hz, 1 H), 7.99 (d, $J$ = 8 Hz, 1 H), 8.02 (d, $J$ = 8.4 Hz, 1 H). $^{13}$C NMR ($CDCl_3$, 400 MHz): $\delta$ 42.4, 53.7, 124.7, 129.8, 130.4, 132.9, 133.7, 148.0, 170.1. HRMS: $m/z$ calcd for $C_9H_8N_1O_4$ (M + 1) 273.9715, found 273.9706.

**Methyl α-Phthalimido-2-nitrophenylacetate (11).** Compound **10 (**3.3 g, 12.1 mmol) was dissolved in dry DMF (90 mL). $K^+$phthalimide (3.7 g, 20.1 mmol) (Aldrich) was then added. The solution was then allowed to stir for 2 h under argon, after which time the DMF was removed under reduced pressure. The remaining brown solid was then suspended in $CH_2Cl_2$ (200 mL) followed by extraction with brine and $H_2O$. The solvent was removed under vacuum. The resulting solid was purified by silica flash chromatography ($CH_2Cl_2$) to result in 3.71 g of **11** as a light yellow solid in 90% yield. $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ 3.80 (s, 3 H), 6.92 (s, 1 H), 7.41 (d, $J$ = 7.8 Hz, 1 H), 7.52 (t, $J$ = 7.4 Hz, 1 H), 7.59 (t, $J$ = 7.6 Hz, 1 H), 7.79−7.81 (2 H), 7.91−7.94 (2 H), 8.15 (dd, $J$ = 1.6 Hz, 8.0 Hz, 1 H). $^{13}$C NMR ($CDCl_3$, 400 MHz): $\delta$ 52.5, 53.4, 123.8, 125.3, 128.7, 129.1, 129.3, 131.3, 133.4, 134.5, 148.4, 166.8, 166.9. HRMS: $m/z$ calcd for $C_{17}H_{12}N_2O_6$ (M + 1) 341.0774, found 341.0787.

**Ethyl (2-Nitrophenyl)glycine (12).** (a) Compound **11** (3.5 g, 10.3 mmol) was placed in a 60:40 solution of concentrated HCl/concentrated acetic acid (17 mL). The solution was refluxed for 4 h. The acid was removed under reduced pressure and the residue dissolved in $H_2O$ (50 mL). The phthalimide was removed via filtration followed by extraction with $CHCl_3$. The aqueous layer was saved and the $H_2O$ removed under reduced pressure to give 1.91 g of 2-nitrophenylglycine hydrochloride as a tan solid in 80% yield. $^1$H NMR (DMSO-$d_6$, 400 MHz): $\delta$ 5.39 (s, 1 H), 7.65 (d, $J$ = 7.6 Hz, 1 H), 7.74 (t, $J$ = 7.4 Hz, 1 H), 7.84 (t, $J$ = 7.6 Hz, 1 H), 8.24 (d, $J$ = 9.0 Hz, 1 H). $^{13}$C NMR (DMSO-$d_6$, 400 MHz): $\delta$ 45.3, 117.4, 118.7, 123.0, 123.8, 126.3, 139.7, 159.3. HRMS: $m/z$ calcd for $C_8H_8N_2O_4$ (M + 1) 197.0562, found 197.0569.

(b) 2-Nitrophenylglycine hydrochloride (1.6 g, 8.01 mmol) was dissolved in EtOH (anhydrous) (75 mL). $p$-Toluenesulfonic acid (monohydrate) (2 equiv) was added and the solution refluxed for 8 h. The solvent was removed under reduced pressure to result in a white solid. The solid was dissolved in a small amount of MeOH followed by precipitation with $Et_2O$ and then filtration and washing with $Et_2O$. The precipitate was dried in vacuo for 12 h to result in 2.59 g of **13** as a white solid, 88% yield. Characterized as the chloride salt. $^1$H NMR ($D_2O$, 400 MHz): $\delta$ 1.18 (t, $J$ = 7.2 Hz, 3 H), 4.28 (m, 2H), 5.73 (s, 1 H), 7.67 (d, $J$ = 7.8 Hz, 1 H), 7.80 (t, $J$ = 8 Hz, 1 H), 7.89 (t, $J$ = 7.4 Hz, 1 H), 8.33 (d, $J$ = 8.2 Hz, 1 H). $^{13}$C NMR ($D_2O$, 400 MHz): $\delta$ 13.5, 55.0, 64.8, 125.8, 126.8, 132.5, 133.5, 136.0, 147.3, 167.7. MS: $m/z$ calcd for $C_{10}H_{13}N_2O_4$ (M + 1) 225, found 225.

***N*-(Methyl 2-nitrophenylglycine)nicotinamide (13).** Compound **12** (2.59 g, 6.73 mmol) was coupled with nicotinoyl chloride and purified using the method described for compound **2**. Purification provided **13**, 1.91 g, as a yellow solid in 82% yield. $^1$H NMR ($CDCl_3$, 300 MHz): $\delta$ 1.21 (t, $J$ = 7.2 Hz, 3 H), 4.23 (m, 2H), 6.23 (d, $J$ = 7.5 Hz, 1 H), 7.39 (m, 1 H), 7.52−7.61 (1 H), 7.68−7.76 (2 H), 8.09 (dt, $J$ = 2.1, 7.8 Hz, 1 H), 8.13 (d, 8.1 Hz, 1 H), 8.74 (d, $J$ = 1.8 Hz, 1 H), 9.0s (s, 1 H). $^{13}$C NMR ($CDCl_3$, 400 MHz): $\delta$ 14.02, 55.7, 62.6, 123.2, 125.5, 128.8, 129.5, 129.7, 131.8, 133.3, 134.2, 134.8, 148.2, 152.4, 164.8, 169.3. HRMS: $m/z$ calcd for $C_{16}H_{15}N_3O_5$ (M + 1) 330.1090, found 330.1078.

**4,5-Dimethoxy-2-nitrobenzaldehyde Oxime Acetate (14).** (a) 6-Nitroveratraldehyde (5.0 g, 23.7 mmol) (Aldrich) and hydroxylamine HCl (1.72 g, 24.9 mmol) were added to a 1:1 mixture of dry pyridine/anhydrous EtOH (150 mL). The solution was refluxed for 8 h followed by removal of the solvent under reduced pressure. The remaining solid was suspended in $CH_2Cl_2$ (400 mL) followed by extraction with 20% sodium bicarbonate and $H_2O$. The organic layer was dried with $MgSO_4$ and filtered and the solvent removed under reduced pressure to produce 4.78 g of 2-nitro-3,4-dimethoxy benzaldehyde oxime as a fibrous yellow solid, 89% yield. $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ 3.98 (s, 1 H), 4.0 (s, 1 H), 7.34 (s, 1 H), 7.64 (s, 1H), 8.80 (s, 1 H). $^{13}$C NMR ($CDCl_3$, 400 MHz): $\delta$ 56.5, 56.6, 106.9, 108.5, 109.0, 121.8, 147.4, 149.7, 152.9. HRMS: calcd for $C_9H_{10}N_2O_5$ (M + 1) 227.0668, found 227.0660.

(b) 4,5-Dimethoxy-2-nitrobenzaldehyde oxime (4.6 g, 20.3 mmol) was dissolved in dry $CH_2Cl_2$ (120 mL) under argon. Dry triethylamine (3.67 mL, 26.4 mmol) was slowly added. The solution was then allowed to stir for 5 min followed by the addition of $Ac_2O$ (2.5 mL, 2.64 mmol) over 10 min. The reaction proceeded for 2 h, after which time $CH_2Cl_2$ (200 mL) was added. The solution was then extracted with $H_2O$. The organic layer was dried with $MgSO_4$ followed by filtration. The solvent was removed under reduced pressure and the remaining yellow solid further purified on a short silica column ($CH_2Cl_2$) to yield 4.3 g of **14** in 73% yield. $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ 2.22 (s, 3 H), 3.98 (s, 1 H), 4.00 (s, 1H), 7.40 (s, 1 H), 7.66 (s, 1 H), 8.99 (s, 1 H). $^{13}$C NMR ($CDCl_3$, 400 MHz): $\delta$ 19.5, 56.5, 56.8, 107.6, 110, 119.8, 141.2, 150.7, 153.0, 153.1, 167.8. HRMS: $m/z$ calcd for $C_{11}H_{12}N_2O_6$ (M + Na) 291.0593, found 291.0588.

**4,5-Dimethoxy-2-nitrobenzylamine (15).** Oxime **14** (3.6 g, 13.4 mmol) was added to dry THF (14 mL). The solution was placed under argon and cooled to 0 °C. Borane (1 M/THF, Aldrich) (80 mL, 80 mmol) was added via syringe. The solution was then refluxed for 24 h. The solution was then cooled to −20 °C, and $H_2O$ (6.0 mL) was added. Aqueous KOH (6.8 M, 3.0 mL) was then slowly added over 20 min. The reaction was then stirred for 3 h at 25 °C. The solution was then added to $CHCl_3$ (550 mL). The $CHCl_3$ was extracted with brine, $H_2O$. The $CHCl_3$ was removed under reduced pressure and the remaining solid purified by silica flash chromatography (10% MeOH, $CH_2Cl_2$, 0.5% DIEA) to result in 1.0 g of **15** as a reddish solid, 35% yield. $^1$H NMR ($CDCl_3$, 400 MHz): $\delta$ 3.91 (s, 3 H), 3.96 (s, 3 H), 4.07 (s, 2 H), 7.03 (s, 1 H), 7.61 (s, 1 H). $^{13}$C NMR ($CDCl_3$, 400 MHz): $\delta$ 44.5, 56.3, 56.5, 107.9, 111.7, 133.6, 140.1, 147.3, 153.3. HRMS: $m/z$ calcd for $C_9H_{12}N_2O_4$ (M + 1) 213.0875, found 213.0884.

**1-(2-Nitrophenyl)ethylamine Hydrochloride (16).** *O*-Nitroacetophenone (1.9 g, 10.9 mmol) and methoxyamine HCl (Aldrich) (10.9 mmol) were added to a 50/50 solution of dry pyridine/anhydrous EtOH (75 mL). The solution was then refluxed for 8 h. The solvent was then removed under reduced pressure and the remaining solid suspended in 200 mL of EtOAc. The EtOAc was then extracted with $H_2O$ and 20% sodium bicarbonate. The organic layer was dried with $MgSO_4$ followed by filtration. The EtOAc was removed under reduced pressure to result in 2.02 g of *o*-nitroacetophenone *O*-methyl oxime (mixture of two diastereomers) as a yellow oil in a yield of 95%. $^1$H NMR ($CDCl_3$, 300 MHz) (mixture, oxime diastereomers, ∼2:1 product ratio): major isomer $\delta$ 2.16 (s, 1 H), 3.18 (s, 3 H), 7.47−8.05 (4 H); minor isomer 2.28 (s, 3 H), 3.71 (s, 3 H), 7.47−8.05 (4 H). MS: $m/z$ calcd for $C_9H_{10}N_2O_3$ (M + 1) 195, found 195.

(b) 2-Nitroacetophenone *O*-methyl oxime (2.12 g, 10.9 mmol) was dissolved in dry THF (8.0 mL) under argon and then cooled to 0 °C. Borane/THF (Aldrich, 1 M (28 mL)) was slowly added followed by reflux for 6 h. The solution was then cooled to −20 °C, and $H_2O$ (2.0 mL) was added. Aqueous 20% KOH, 2.0 mL was then added over 20 min followed by reflux, 2 h. The solution was added to $CH_2Cl_2$ (250 mL) followed by extraction with brine and $H_2O$. The organic layer was then dried with $MgSO_4$ and the filtered solvent removed under reduced pressure. The remaining oil was suspended in 30 mL of $CH_2Cl_2$ and then acidified with concentrated HCl (1.5 mL) with stirring. The resulting precipitate was separated by filtration and washed with $Et_2O$. The solid was dried in vacuo to result in 1.2 g of **16** in 50% yield. $^1$H NMR (DMSO-$d_6$, 300 MHz) $\delta$ 1.60 (d, $J$ = 6.0 Hz, 3 H), 4.76 (q, $J$ = 5.7 Hz, 1 H), 7.63 (t, $J$ = 7.8, 1 H), 7.63 (t, $J$ = 7.2 Hz, 1 H), 7.84 (t, $J$ = 7.2 Hz, 1 H), 8.00 (d, $J$ = 8.1 Hz, 1 H), 8.08 (d, $J$ = 7.8 Hz, 1 H) $^{13}$C NMR (DMSO-$d_6$, 300 MHz) $\delta$ 20.5, 45.4, 124.5, 128.1, 129.5, 133.8, 133.9, 148.0. HRMS: *m/z* calcd for $C_8H_{10}N_2O_2$ (M + 1) 167.0821, found 167.0827.

**Preparation of NADase.** Pig brain NADase (Sigma) (2.5 g, 0.009 units/mg) was placed in $H_2O$ (200 mL, Trizma 50 mM (pH = 7.6 at 25 °C) at 4 °C along with antifoam B (0.5 mL, Sigma). The mixture was placed in a Waring blender at high speed, 15 s, followed by low speed, 45 s. The solution was then placed in a vacuum flask and the solution degassed. The mixture was then centrifuged at 15 000 rpm, 20 min, 4 °C. The supernate was discarded and the enzyme pellet *thoroughly* resuspended in $H_2O$ (40 mL, 0.05 M Trizma (pH = 7.6 at 25 °C) using an all-glass homogenizer. This mixture was then centrifuged at 15 000 rpm, 4 °C, for 20 min. The supernate was discarded and the pellet resuspended in 40 mL, 50 mM Trizma, pH = 7.6 (at 25 °C) buffer using an all glass homogenizer. This suspension was then sonicated (Branson 450 sonicator with emersion tip, 70% duty cycle) for three 1 min intervals at 4 °C. The enzyme suspension was used immediately after sonication.

***N*-(4,5-Dimethoxy-2-nitrobenzyl)-*β*-nicotinamide Adenine Dinucleotide Phosphate (18).** Compound **2** (0.3 g, 0.5 mmol) was added to $H_2O$ (100 mL) buffered with Trizma (50 mM, pH = 7.6). The mixture was heated to 80 °C and then allowed to cool unassisted to 40 °C while stirring. NADase enzyme preparation (40 mL) and then NADP (0.45 g, 0.059 mmol) (monosodium salt, Sigma) were added. The mixture was placed in a shaker at 37 °C and 250 rpm. After 20 min, the solution was adjusted to pH = 7.3 at 37 °C with 3 N HCl. The reaction mixture was then allowed to continue shaking for an additional 7.0 h. The reaction was stopped via the addition of 10% trichloroacetic acid (aqueous) (30 mL) at 4 °C. The mixture was then placed in a centrifuge and spun for 20 min at 15 000 rpm. The supernate was saved, and the pellets were discarded. The remaining solution was extracted with $Et_2O$ (3 × 40 mL) and $CH_2Cl_2$ (3 × 40 mL). The aqueous layer was saved and the $H_2O$ removed via lyophilization to result in a yellow powder. The remaining solid was added to $H_2O$ (15 mL) and then filtered (MSI, 0.45 micron, acetate). **Purification.** Purification was accomplished via HPLC using a Vydec semipreparative $C_{18}$ column. Isochratic elution conditions were applied to the above solution utilizing a tandem pump configuration, 260 nm UV detection. Initial conditions: flow = 2.0 mL/min; pump A, 10% MeOH, $NaPO_4H_3$ (0.1 M, pH = 4.8). Compound **18** eluted at 18.0 min. Samples were pooled and lyophilized. The remaining powder was dissolved in $H_2O$ (1 mL) and reinjected under the described HPLC conditions so as to maximize purity. Following lyophilization, **18** is obtained in 9% yield (based upon UV absorbance at 355 nm, $\epsilon$ = 5000 $cm^{-1}$ $M^{-1}$) as a light yellow powder. $^1$H NMR ($D_2O$, 400 MHz): $\delta$ 3.63 (s, 3 H), 3.67 (s, 3 H), 4.03−4.46 (9 H), 4.68 (m, 1 H), 4.8 (2 H), 5.73 (d, $J$ = 6 Hz, 1 H), 6.09 (d, $J$ = 6.1 Hz, 1 H), 7.39 (s, 1 H), 8.03 (s, 1 H), 8.09 (t, $J$ = 7.6 Hz, 1 H), 8.21 (s, 1 H), 8.73 (d, $J$ = 7.6 Hz, 1 H), 8.98 (d, $J$ = 6.4 Hz, 1 H), 9.48 (s, 1 H). ESI MS: *m/z* calcd for $C_{30}H_{38}N_8O_{21}P_3$ (M + 1) = 939, found 939; $C_{30}H_{36}N_8O_{21}P_3$ (M − 1) = 937; found 937. $\lambda_{max}$ ($H_2O$, pH = 7.3) = 249 nm. Compound **18** purity >95%, as determined by analytical HPLC (YMC ODS-AQ, 250 × 6.0 mm, S-$\mu$m, 120 Å, 260 nm detection, flow = 1 mL/min) using the gradient conditions stated for compound **19**.

***N*-(2-Nitrophenylglycine)-*β*-nicotinamide Adenine Dinucleotide Phosphate (19).** Compound **4** (1.6 g, 4.6 mmol) was placed in $H_2O$ (20 mL, 0.075 mM Trizma, pH = 7.3) and 6 N NaOH slowly added until the solution cleared. $H_2O$ (14 mL, 0.075 mM Trizma, pH = 7.3) was then added and the solution adjusted to pH = 7.6 with 6 N HCl. Nadase enzyme suspension, 8 mL (see above procedure), was then added followed by NADP (0.188 g, 0.025 mmol), Sigma monosodium salt. This solution was placed in a shaker at 37 °C, 250 rpm for 20 min, after which the solution was adjusted to pH = 7.3. Every 2 h over an 8 h period the enzyme was removed via ultracentrifugation (15 000 rpm, 4 °C, 10 min). Fresh enzyme (10 mL) was then added to the supernate. After 8 h, the reaction mixture was then taken and centrifuged at 15 000 rpm for 25 min. The supernate was saved and kept at 4 °C. Concentrated HCl was then added, and the excess nicotinamide **4** was precipitated. (After rinsing with $H_2O$ and a small amount of ethanol ∼85% of starting material was recovered as the HCl salt.) The remaining solution was then extracted $CH_2Cl_2$ (4 × 10 mL). The aqueous layer was saved and lyophilized down to a yellow solid. **Purification.** The remaining solid was added to 10 mL of $H_2O$ and then filtered (MSI, acetate, 0.45 $\mu$m). Purification was accomplished via HPLC using a Vydec semipreparative $C_{18}$ column. Gradient elution conditions were applied to utilizing a tandem pump configuration, 260 nm UV detection. Initial conditions: flow = 2.0 mL/min; pump A, 0.1 M $NaPO_4H_3$ buffer, pH = 4.8; pump B, flow = 0 mL/min, 50% MeOH, 50% 0.1 M $NaPO_4H_3$ buffer, pH = 4.8. At a constant flow rate of 2.0 mL/min: time 0 → 21 min (0% B → 18% B); time 21 → 42 min (18% B → 100% B); time 42−55 min (100% B). Product elution time = 30 min. Samples were pooled and lyophilized followed by a reinjection (∼1 mL) under the above HPLC conditions to optimize purity. Following lyophilization, caged NADP **19** was obtained in 9% yield (based upon UV absorbance at 260 nm). $^1$H NMR ($D_2O$, 400 MHz): $\delta$ 4.08−4.51 (9 H), 4.96 (m, 1 H), 5.95 (s, 1 H), 5.96 (d, $J$ = 5.2 Hz, 1 H), 6.04 (d, $J$ = 6 Hz, 1 H), 7.98−8.02 (2 H), 8.08 (t, $J$ = 6.8 Hz, 1 H), 8.30 (s, 1 H), 8.38 (d, $J$ = 8 Hz, 1 H), 9.02 (d, $J$ = 6 Hz, 1 H), 9.19 (s, 1 H). ESI MS: *m/z* calcd for $C_{29}H_{34}N_8O_{21}P_3$ (M + 1) = 923; $C_{29}H_{32}N_8O_{21}P_3$ (M + 1) = 921. $\lambda_{max}$ ($H_2O$, pH = 7.3) = 259 nm. Compound **19** purity >95%, as determined by analytical HPLC (YMC ODS-AQ, 250 × 6.0 mm, S-$\mu$m, 120 Å, 260 nm detection, flow = 1 mL/min) using the gradient conditions stated for compound **19**.

***N*-(4-(1-Amino)ethyl-3-nitrobenzoate)-*β*-nicotinamide Adenine Dinucleotide Phosphate (20).** Compound **3** (2.75 g, 7.8 mmol) was placed in $H_2O$ (20 mL) and 6 M NaOH (∼2.6 mL) slowly added until the solution cleared. This solution was then added to $H_2O$ (56 mL, Trizma pH = 7.6, 50 mM). The solution was then readjusted to pH = 7.6. Nadase enzyme preparation (10 mL) was added followed by NADP (0.3 g, 0.039 mmol) (monosodium salt, 98%, Sigma). The solution was again adjusted to pH = 7.6. The mixture was then placed in a shaker at 37 °C, 250 rpm. After 20 min, the mixture should attain pH = 7.3 at 37 °C. Every 2 h over an 8 h period the enzyme was removed via ultracentrifugation (15 000 rpm, 4 °C, 10 min). Fresh enzyme (10 mL) was then added to the supernate. After 8 h, the reaction mixture was then taken and spun in a centrifuge at 15 000 rpm for 25 min. The supernate was saved and kept at 4 °C. Concentrated HCl was then added and the excess nicotinamide **3** precipitated. (After rinsing with $H_2O$ and a small amount of ethanol ∼90% of starting material was recovered as the HCl salt.) The remaining solution was then extracted with $CH_2Cl_2$ (4 × 10 mL). The aqueous layer was saved and lyophilized down to a yellow solid. **Purification.** The remaining solid was added to $H_2O$ (12 mL) and then filtered (MSI, acetate filter, 0.45 $\mu$m). Purification was accomplished via HPLC using the gradient conditions specified for compound **19**. Product elution time = 30 min. Following lyophilization, 7% caged NADP **20** (based upon UV absorbance at 260 nm) was obtained as a white powder. $^1$H NMR ($D_2O$, 400 MHz): $\delta$ 4.00−4.39 (9 H), 4.89 (m, 1 H), 5.34 (q, $J$ = 6.8 Hz, 1 H), 5.92 (d, $J$ = 6 Hz, 1 H), 5.95 (d, $J$ = 5.2 Hz, 1 H),

7.54 (d, $J = 8.4$ Hz, 1 H), 7.90 (dd, $J = 8$ Hz, 2 Hz, 1 H), 8.01 (s, 1 H), 8.07 (t, $J = 6.8$ Hz, 1 H), 8.19 (s, 1 H), 8.23 (s, 1 H), 8.67 (d, $J = 8$ Hz, 1 H), 8.99 (d, $J = 6.4$ Hz, 1 H), 9.27 (s, 1 H). ESI MS: *m*/*z* calcd for $C_{30}H_{36}N_8O_{21}P_3$ (M + 1) = 937, found 937; $C_{30}H_{34}N_8O_{21}P_3$ (M − 1) = 935, found 935. $\lambda_{max}$ ($H_2O$, pH = 7.3) = 258 nm. Compound **20** purity >95%, as determined by analytical HPLC (YMC ODS-AQ, 250 × 6.0 mm, S-$\mu$m, 120 Å, 260 nm detection, flow = 1 mL/min) using the gradient conditions stated above.

**Acknowledgment.** We thank Prof. Joseph Kraut for continuing support of this work and the members of the Scripps Research Institute Mass Spectral Facility for outstanding assistance.

**Supporting Information Available:** Spectroscopic information including $^1$H NMR and COSY spectra of **18**, **19**, and **20** as well as $^1$H NMR and selected $^{13}$C NMR of most compounds. UV and mass spectral characterization of byproducts are also included. This material is available free of charge via the Internet at http://pubs.acs.org.

JO9917846