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Nicotinic Acid Adenine Dinucleotide Phosphate Analogues Containing Substituted Nicotinic Acid: Effect of Modification on Ca^{2+} Release†

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Analogues of nicotinic acid adenine dinucleotide phosphate (NAADP) with substitution at either the 4- or the 5-position position of the nicotinic acid moiety have been synthesized from NADP enzymatically using *Aplysia californica* ADP-ribosyl cyclase or mammalian NAD glycohydrolase. Substitution at the 4-position of the nicotinic acid resulted in the loss of agonist potency for release of Ca^{2+}-ions from sea urchin egg homogenates and in potency for competition ligand binding assays using [^{32}P]NAADP. In contrast, several 5-substituted NAADP derivatives showed high potency for binding and full agonist activity for Ca^{2+} release. 5-Azido-NAADP was shown to release calcium from sea urchin egg homogenates at low concentration and to compete with [^{32}P]NAADP in a competition ligand binding assay with an IC_{50} of 18 nM, indicating that this compound might be a potential photoprobe useful for specific labeling and identification of the NAADP receptor.

Introduction

Nicotinic acid adenine dinucleotide phosphate (NAADP, **1**)[a] (Chart 1) was identified as a metabolite of NADP, which at low concentration was shown to cause Ca^{2+} release from internal calcium ion stores in sea urchin eggs.[1] NAADP-mediated Ca^{2+} release was subsequently shown to be pharmacologically distinguishable from calcium release mediated by either inositol-1,4,5-triphosphate ($InsP_3$) or by cyclic ADP-ribose (cADPR). Additionally, NAADP mobilized Ca^{2+} from a different subcellular location than does cADPR or $InsP_3$.[2] These observations imply that NAADP releases calcium ion after binding to a unique receptor.[3−6] NAADP is active in many organisms including mollusks, plants, and mammals.[4] In vertebrate tissue, NAADP-mediated calcium release has been observed in pancreatic acinar cells,[7] cultured human T lymphocytes,[8] brain,[9] heart,[10] kidney,[11] and liver cells.[12] In some of these systems, NAADP has met the criteria for establishing it as an intracellular second messenger.[3,13]

Despite its physiological importance, the structural features of NAADP which are required for high potency calcium release are not completely understood. Lee et al. defined the pyridine-3-carboxylate, the adenosyl 2′-phosphate, and portions of the purine as important structural determinants.[14] Billington further defined the requirements of 3-substituted pyridine modification on agonist activity.[15] Simple pyridinium-3-carboxylate salts[16,17] and pyridoxalphosphate-6-azophenyl-2,4-disulfonate (PPADS)[18] are reported to be low potency antagonists. A potent and selective NAADP antagonist, Ned-19, was developed recently using virtual screening[19] and has been applied to studies of the NAADP receptor.[20] Continued progress in the development of chemical probes for NAADP-mediated signaling and characterization of the NAADP receptor require a better understanding of the structure−activity relation (SAR) of NAADP-mediated calcium ion release. To this end, we have synthesized a series of nicotinic acid substituted NAADP derivatives using a chemoenzymatic synthesis[21,22] and characterized their ability both to mobilize calcium-ion via the NAADP receptor in sea urchin egg homogenates and to compete with NAADP in competition ligand binding assay.

Chemistry

Because the enzymes *Aplysia californica* ADP-ribosyl cyclase and mammalian NAD glycohydrolase are known to catalyze the exchange of the nicotinamide group of NADP with nicotinic acid (Scheme 1),[21,22] we obtained nicotinic acid derivatives with substituents at either position 4 or 5 and used them as substrates (Table 1) for the enzyme-catalyzed pyridine base exchange reaction to produce the corresponding NAADP analogues. The general reaction for the base exchange is shown in Scheme 1.

4-Substituted Nicotinic Acids. We sought to obtain 4-substituted nicotinic acid derivatives with alkyl, amino, or aryl groups at the 4-position. The simple 4-methylnicotinic acid (**2a**) as well as 4-aminonicotinic acid (**2b**) were both available

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[a] Abbreviations: cADPR, cyclic adenosine diphosphate ribose; DIPEA, diisopropylethylamine; DMF, *N*,*N*-dimethylformamide; ESI, electrospray ionization mass spectrometry; EtOAc, ethyl acetate; EtOH, ethyl alcohol; Fluo-3, refers to the fluorescent indicator Ca^{2+} dye developed by Dr. Roget Y. Tsien; HPLC, high pressure liquid chromatography; HRMS, high mass resolution mass spectrometry; InsP3, inositol-1,4,5-triphosphate; LC-mass spec, liquid chromatography mass spectrometry; NAADP, nicotinic acid adenine dinucleotide phosphate; NADase, nicotinamide adenine dinucleotide glycohydrolase; PPADS, pyridoxalphosphate-6-azophenyl-2,4-disulfonate; SAR, structure−activity relation; TCA, trichloroacetic acid; TEA, triethylamine; TES, triethylsilyl group; THF, tetrahydrofuran; TLC, thin layer chromatography; Tris, tris-(hydroxymethyl)aminomethane; UV, ultraviolet light;

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Scheme 1. Synthesis of NAADP Analogues Using the Enzyme Catalyzed Pyridine Base Exchange Reaction

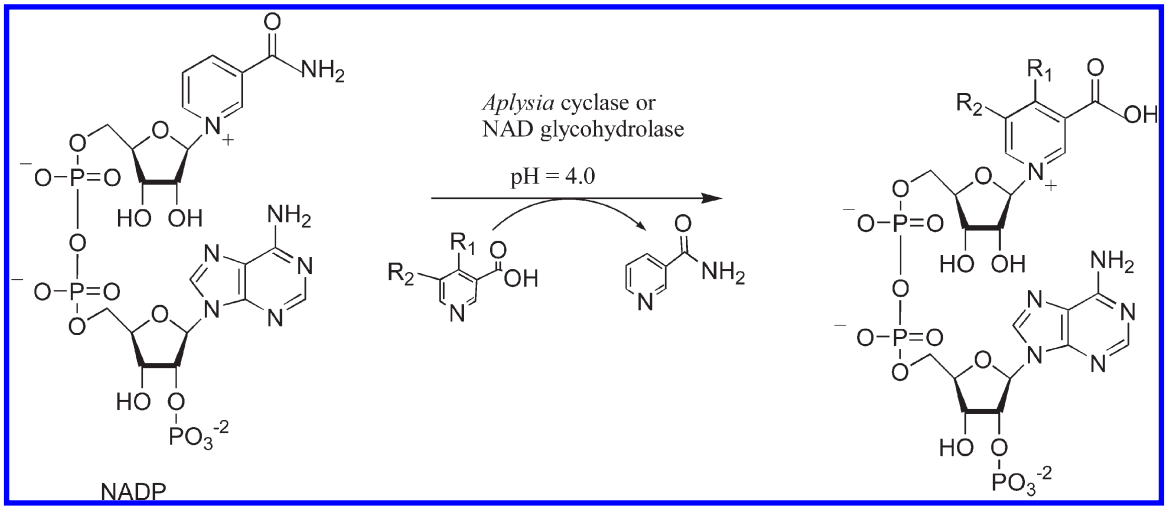
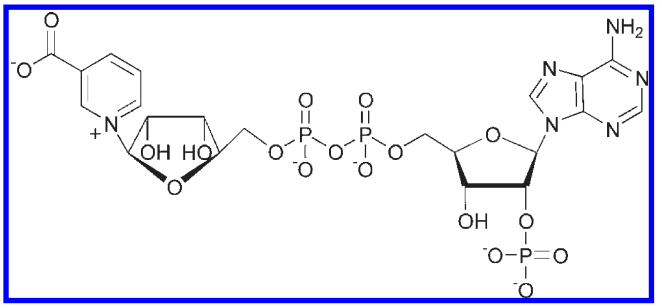


Chart 1. Nicotinic Acid Adenine Dinucleotide Phosphate (NAADP), 1

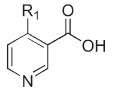
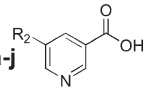
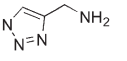


commercially. 4-*n*-Butylnicotinic acid and 4-phenylnicotinic acid were synthesized by adding either *n*-butyllithium or phenyllithium respectively to pyridyl-3-oxazolines derived from nicotinic acid (Scheme 2).[23] Addition of the organolithium reagent was shown to favor the formation of the 1,4-dihydropyridine-3-oxazoline, which was easily oxidized by oxygen in air to obtain the 4-substituted pyridine-3-oxazoline. Deprotection results in the isolation of the desired 4-substituted nicotinic acids.[24]

5-Substituted Nicotinic Acids. The simple derivatives 5-methylnicotinic acid (**3a**) and 5-aminonicotinic acid (**3b**), 5-bromonicotinic acid (**3c**), and pyridine-3,5-dicarboxylic acid (**3d**) were purchased. For the synthesis of 5-substituted nicotinic acid derivatives, we started with commercially available and inexpensive 5-bromonicotinic acid (**3c**) and protected the carboxylic acid by its conversion into the oxazoline group (**4**). Transmetalation of **4** was expected to be difficult because of competing 4-addition reaction.[23] Therefore we utilized intermediate **4** in palladium catalyzed C−C coupling reactions with appropriate coupling partners to produce 5-substituted pyridine-3-oxazolines.

The Sonogashira coupling has previously been used successfully to produce ethynylpyridines,[25,26] and we expected that cross-coupling of terminal acetylenes with **4** (Scheme 3) would provided an approach to 5-ethynylnicotinic acid (**3e**). Coupling of **4** with commercially available triethylsilylacetylene (TES-acetylene), under conditions using a mixed aqueous−organic solvent and sodium carbonate at 90 °C, led to formation of disubstituted acetylene **5** instead of the desired product as shown in Scheme 3. Formation of **5**

Table 1. 4-Substituted Pyridine-3-carboxylic Acids (**2a**−**d**) and 5-Substituted Pyridine-3-carboxylic Acids (**3a**−**j**) Obtained and Evaluated As Potential Substrates in the Chemoenzymatic Synthesis Designed to Produce Novel Pyridinium Substituted NAADP Derivatives

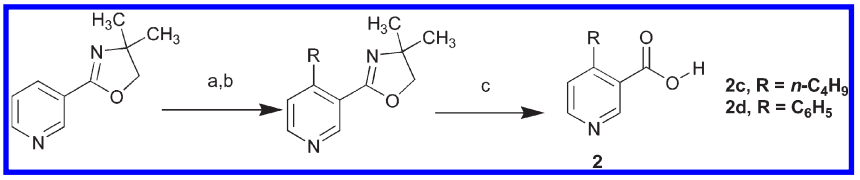
Structure	Cmpd	Substituent	Base-exchange product detected
2a-d 	**2a**	-NH_2	no; yes for methyl ester
	2b	-CH_3	yes
	2c	-*n*-C_4H_9	yes
	2d	-C_6H_5	yes
3a-j 	**3a**	-NH_2	yes
	3b	-CH_3	yes
	3c	-Br	no
	3d	-CO_2H	yes
	3e	-C≡CH	no
	3f	-$CH{=}CH_2$	no
	3g	-CH_2CH_3	yes
	3h	-C_6H_5	no
	3i	-N_3	yes
	3j		no

probably occurred because in the presence of aqueous base and high temperature the triethylsilyl group was removed from initially formed **6**, and the free acetylene liberated in situ reacted with another molecule of **4**.

When the reaction was conducted at room temperature and by using a phase transfer catalyst with a minimum amount of water in the reaction,[27] we were able to obtain **6** in high yield. The silyl group in **6** was removed by treatment with excess aqueous potassium carbonate,[28] followed by acid hydrolysis of the oxazoline group[29] to give free acid derivative **3e** (Scheme 3).

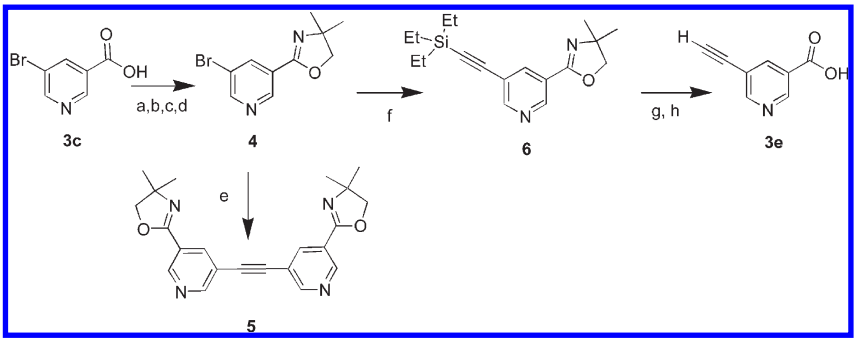
Compound **3f** was obtained by partial reduction of **3e** in the presence of poisoned Lindlar's catalyst.[30] The acetylene

Scheme 2. Addition of Organolithium Reagents to Pyridine-3-oxazoline[a]



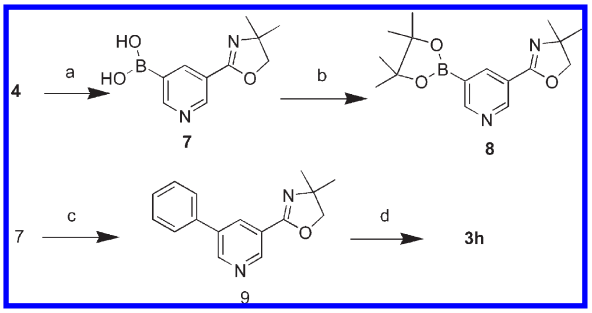
[a] Conditions and reagents: (a) C_6H_5Li or n-C_4H_9Li, −78°; (b) air, 0° 3 h; (c) 3 N HCl/acetic acid 95°/36 h.

Scheme 3[a]



[a] Conditions and reagents: (a) $SOCl_2$, reflux, 18 h; (b) 2-amino-2-methylpropanol, 48 h; (c) $SOCl_2$, 24 h; (d) diisopropylethylamine (DIPEA), 60 °C, 24 h; (e) $Pd(Ph_3)_4$, Na_2CO_3, CuI, TES-acetylene, dimethylformamide (DMF)/ethyl alcohol (EtOH)/H_2O at 90° for 1 h; (f) $Pd(OAc)_2$, PPh_3, benzyltributylammonium bromide, TES-acetylene, DIPEA and water, room temperature, 1 h; (g) K_2CO_3, tetrahydrofuran (THF)/ CH_3OH, room temperature, 1 h; (h) 3 N HCl, AcOH, 95° 1 h.

Scheme 4[a]



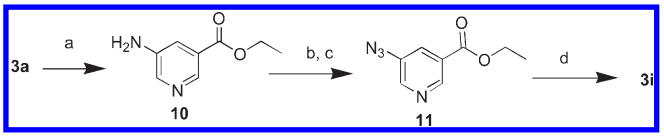
[a] Conditions and reagents: (a) toluene/THF, $(i\text{-}C_3H_7O)_3B$, then n-BuLi, −70°; (b) $(CH_3)_2COHCOH(CH_3)_2$, Ph-CH_3, reflux; (c) $Pd(Ph_3)_4$, Na_2CO_3,, C_6H_5-Br, DMF and EtOH 90° for 4 h; (d) 3N HCl, acetic acid, 95° 36 h.

of compound **3e** was completely reduced to an ethyl group using ammonium formate in presence of Pd on carbon to obtain 5-ethylnicotinic acid (**3g**).

For the synthesis of the 5-arylnicotinic acids, Pd-catalyzed Suzuki reaction between aryl halides and boronic acids has been used (Scheme 4).[26] We synthesized **9** by means of Suzuki coupling between the boronate of oxazoline substituted nicotinic acid **7** and phenyl bromide. The boronate of the protected nicotinic acid could not be completely characterized by itself because it was a mixture of several boronate forms (boronic acids and boronic esters) that have been reported to exist.[31] Characterization of the boronate was accomplished by converting the boronate into its pinacol ester (**8**).[31]

The borate **7** was coupled to phenyl bromide in the presence of Pd catalyst and aqueous base at 90 °C to give the C−C coupled product **9**. The deprotection of oxazoline under acidic conditions gave 5-phenylnicotinic acid **3h** (Scheme 4).

Scheme 5[a]



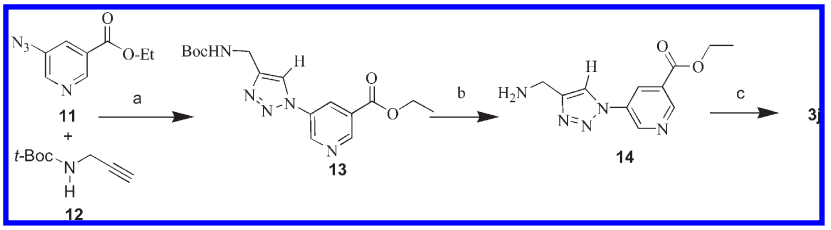
[a] Conditions and reagents: (a) EtOH/HCl; (b) $NaNO_2$/HCl; (c) NaOAc/NaN_3; (d) CH_3OH/NaOH/H_2O.

5-Azidopyridine-3-carboxylate (**3i**) was an important precursor both to subsequently establish the SAR of NAADP derivatives and for developing high potency photoaffinity probes for receptor identification.[32] Literature provided the precedent of diazotization reaction, followed by nucleophilic displacement by sodium azide.[33,34] This approach was used to synthesize 5-azidonicotinic acid **3i** starting from 5-aminonicotinic acid **3a** (Scheme 5). We started with protection of the acid group in 5-aminonicotinic acid (**3a**) with a hydrophobic ethyl ester (**10**), followed by conversion of the amine into an azide using diazotization chemistry to give **11**.[35] The hydrophobic ester group used for acid protection aided in extracting the compound into the organic phase, while the salts from the reaction partitioned into the aqueous phase. 5-Azidonicotinic acid ethyl ester **11** was then subjected to alkaline hydrolysis to give 5-azidonicotinic acid **3i**.

Nicotinic acid derivative **3j** was synthesized by click chemistry involving [1,3] dipolar cycloaddition reaction[36] between the 5-azidonicotinate ethyl ester (**11**) and *tert*-butyl prop-2-ynylcarbamate **12** (Scheme 6). *tert*-Butyl prop-2-ynylcarbamate (**12**) was prepared according to procedure described by Holmes et al. (2003).[25]

The copper catalyzed cycloaddition resulted in formation of 1,4-disubstituted triazole ring at position 5 of nicotinic acid (**13**). Use of copper catalyst brings regioselectivity to the reaction and catalyzed the formation of an adduct between the azide and the acetylene moiety producing only the 1,4-disubstituted isomer.

Scheme 6[a]



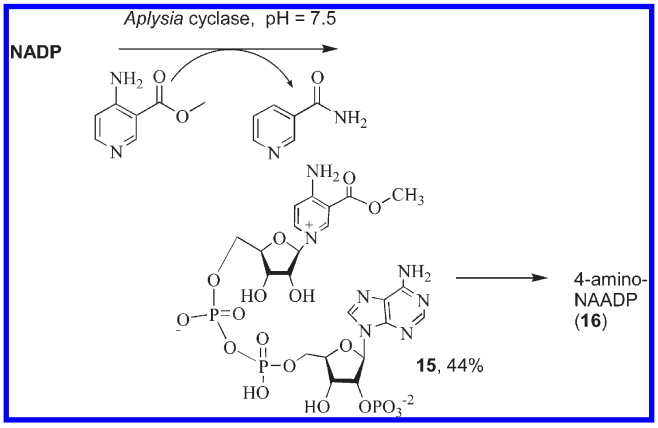
[a] Conditions and reagents: (a) DIPEA, CuI, 12 h; (b) trifluoroacetic acid; (c) 2 N NaOH in H_2O/CH_3OH.

The competing reaction pathway producing the 1,5-disubstitution cycloaddition product is thereby eliminated.[37] Deprotection in strong anhydrous acid produced ester **14**, which was saponified to the nicotinic acid derivative **3j**.

Enzyme Catalyzed Base Exchange. NAADP derivatives were synthesized from pyridine-3-carboxylic acid derivatives and NADP either by using the enzymatic activity of *Aplysia* ADP-ribosyl cyclase or a mammalian NAD glycohydrolase (Scheme 1). We relied primarily on the *Aplysia* ADP-ribosyl cyclase as the catalyst because it was available as a stable, soluble, and well-characterized enzyme. In cases where the *Aplysia* ADP-ribosyl cyclase failed to produce the expected exchange product, mammalian enzymes were also evaluated. A high concentration of a nucleophilic pyridine base is required for high yields of the exchange product. In a typical reaction, NADP was treated with about a 30-fold excess of the pyridine derivative and the enzyme catalyst at pH 4.0. Under these conditions, base exchange competes successfully with both hydrolysis and cyclization reactions, side reactions which destroy the substrate NADP by forming either ADP-ribose or cADPR byproducts. The control of pH is important because it is the un-ionized pyridine derivative that is the substrate for the base exchange reaction. An optimal pH must be maintained to provide high concentrations of a un-ionized pyridine base. The exchange reactions were monitored by following the disappearance of the limiting substrate, NADP. In this assay, NADP present in the mixture was reduced using glucose-6-phosphate in the presence of glucose-6-phosphate dehydrogenase to give NADPH. The NADPH that was produced showed absorbance at 340 nm, without interference from any other reactant or biproduct.[3] When the exchange reaction was complete, the catalyst was removed by ultrafiltration (membrane cut off 10 kDa). The pH of the catalyst free filtrate was adjusted to 7, and dinucleotides present in the mixture were purified by anion-exchange chromatography.

Methylated nicotinic acids such as **2b** or **3b** could easily be exchanged into NADP to result in the formation of NAADP derivatives **17** and **21**. 5-Aminonicotinic acid (**3a**) was similarly an excellent exchange substrate, and the exchange product 5-amino-NAADP (**20**) could be isolated in greater than 40% yield after purification.

We tried to exchange commercially available 4-aminonicotinic acid (**2a**) into NADP at pH 4.0 to enzymatically synthesize the 4-amino-NAADP (**16**)[38] but were unsuccessful in detecting any pyridine dinucleotide derivatives as products. The likely explanation is that **2a**, a vinylogous amidine, is significantly more basic than is **3a** and is largely protonated and cationic under the usual conditions of the reaction at pH 4. To circumvent this difficulty, we synthesized the methyl ester of 4-aminonicotinic acid using Fischer esterification and used the 4-aminonicotinic acid methyl ester as the cosubstrate for the exchange at pH 7.5. Under these conditions, we obtained exchange product **15**. Use of this higher pH increased the concentration of the unprotonated and un-ionized 4-aminonicotinic acid methyl ester, and hence it became more available to the active site of NAD glycohydrolase. The 4-amino-NAADP methyl ester was then subjected to treatment with triethylamine and water to hydrolyze the ester and give the target compound 4-amino-NAADP (**16**).

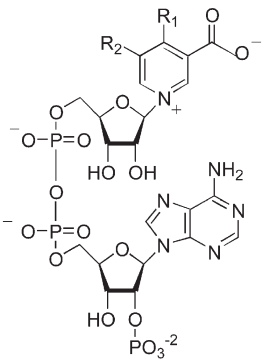


Base exchange reaction with 4-*n*-butylnicotinic acid (**2c**) or 4-phenyl nicotinic acid (**2d**) worked well to produce NAADP derivatives **18** and **19**. Enzyme catalyzed base exchange was surprisingly more selective when substituents were placed in the 5 position. Neither 5-phenylnicotinic acid (**3h**) nor bromonicotinic acid (**3c**) formed detectable base-exchanged product. Similarly, **3e**, **3f**, and **3j** failed to react to form a substituted NAADP. It is well appreciated that electron withdrawing substituents deactivate the pyridine ring toward base exchange. Steric demands on disubstituted pyridines are less well understood, although it is apparent from our work that large substituents are better tolerated at the 4-position than at the 5-position.

Interestingly, 5-substitutions like azide and carboxylate were tolerated as cosubstrates for exchange reactions, producing reasonable yields of the corresponding NAADP analogues. In cases where the exchange reaction catalyzed by the *Aplysia* ADP-ribosyl cyclase was found to fail, we also tried NAD glycohydrolase enzymes from porcine brain and bovine spleen, but even when using the mammalian enzyme as a catalyst, the result was the same.

The results of enzyme catalyzed base exchange reactions between NADP and our set of synthetic nicotinic acids are summarized in Table 2. We also observed that all three enzymes behaved very similarly, and the outcome of exchange reactions using different substrates was the same for each of the catalysts. This suggests that the active site of the

Table 2. Result of the Pyridine Base Exchange Reaction

Structure	R_1	R_2	#	Name	%Yield
	$-NH_2$	-H	**16**	4-amino-NAADP	44
	$-CH_3$	-H	**17**	4-methyl-NAADP	43
	$-n-C_4H_9$	-H	**18**	4-*n*-butyl-NAADP	60
	$-C_6H_5$	-H	**19**	4-phenyl-NAADP	51
	-H	$-NH_2$	**20**	5-amino-NAADP	43
	-H	$-CH_3$	**21**	5-methyl-NAADP	59
	-H	$-CO_2H$	**22**	5-carboxy-NAADP	32
	-H	$-CH_2CH_3$	**23**	5-ethyl-NAADP	59
	-H	$-N_3$	**24**	5-azido-NAADP	61

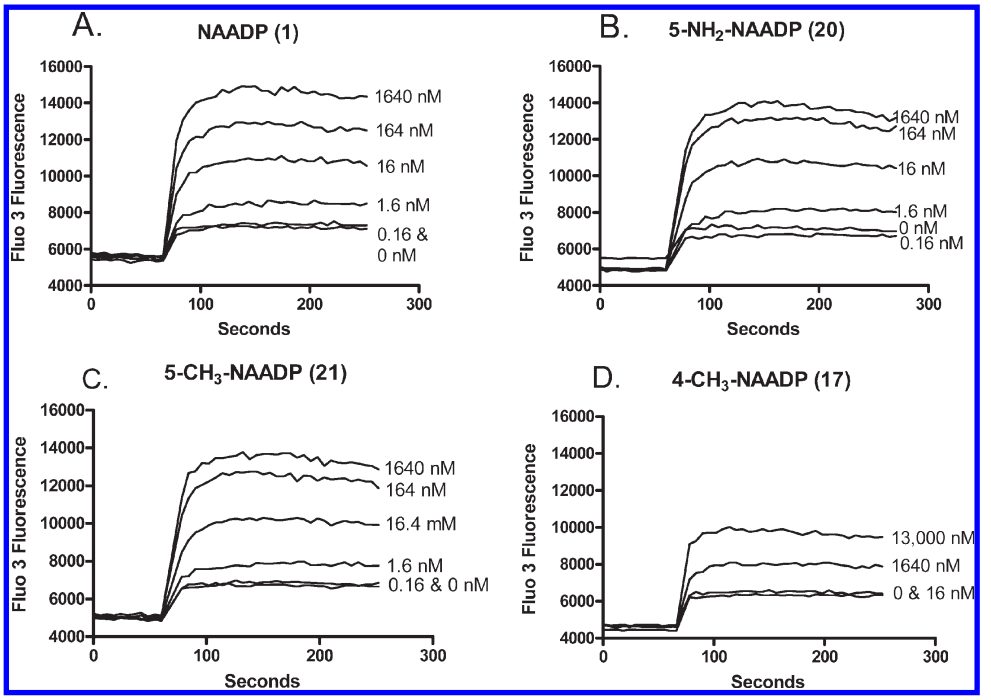


Figure 1. Fluorimetric calcium release traces from sea urchin egg homogenates induced by the addition of increasing concentrations of NAADP and three selected NAADP analogues. Calcium-ion concentration was measured fluorimetrically using the calcium sensitive dye Fluo-3. The effect of the addition of increasing concentrations of NAADP (**1**) (A), 5-NH_2-NAADP (**20**) (B), 5-CH_3-NAADP (**21**) (C), and 4-CH_3-NAADP (**17**) (D) on fluorescence are shown.

three enzymes, as judged by the specificity of base exchange reactions, is very similar.

Biological Activity

Calcium Release Properties of Compounds 16−24. When tested for calcium ion-releasing activity in vitro, NAADP (**1**) and its analogues **16**−**24** were shown to elicit calcium release from sea urchin egg homogenates. Figure 1 shows the effect of treatment of calcium-ion loaded microsomes with varying concentrations of NAADP (**1**), 5-NH_2-NAADP (**20**), 5-CH_3-NAADP (**21**), and 4-CH_3-NAADP (**17**). Each of these compounds induced a rapid release of calcium-ions which reached a concentration dependent plateau within a few seconds. Compounds **20** and **21** induce calcium-ion release at concentrations as low as 2 nM. 4-CH_3-NAADP (**17**) was shown to be significantly less potent than was the 5-isomer **21**. The maximal calcium concentration attained after treatment was found to increase in a concentration dependent manner. Saturating concentrations of compounds **20** and **21** elicited the same amount of calcium release as did the full agonist NAADP. Figure 2 shows concentration−response curves for compounds **16**−**24** compared to

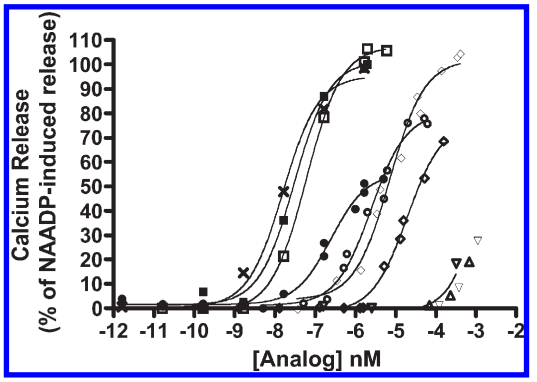


Figure 2. Concentration−response curves depicting maximum Ca^{2+} concentration attained after treatment of calcium loaded sea urchin egg homogenates by the addition of varying concentrations of NAADP (**1**) (■), 5-NH_3-NAADP (**20**) (□), 5-CH_3-NAADP (**21**) (×), 5-CH_2CH_3-NAADP (**23**) (●), 5-N_3-NAADP (**24**) (○), 4-NH_3-NAADP (**16**) (light ◇), 4-CH_3-NAADP (**17**) (dark ◇), 4-*n*-butyl-NAADP (**18**) (light ▽), 4-phenyl-NAADP (**19**) (dark △), and 5-CO_2H-NAADP (**22**) (dark ▽). Each plotted point represents the mean of a minimum of three determinations.

Table 3. EC_{50} Values for Ca^{2+} Release Induced by NAADP (**1**) and NAADP Analogues (**16**−**24**) Measured Fluorometrically from Ca^{2+} Loaded Sea Urchin Egg Homogenates

compd	EC_{50} ± SD, nM (*n*)	Fold increase in EC_{50}
NAADP (**1**)	18.7 ± 6.9 (19)	1
5-amino-NAADP (**20**)	27.4 ± 13.9 (9)	1.5
5-methyl-NAADP (**21**)	43.0 ± 25.3 (11)	2.3
5-ethyl-NAADP (**23**)	352 ± 219 (3)	18
5-azido-NAADP (**24**)	1689 ± 1349 (9)	90
4-amino-NAADP (**16**)	4413 ± 2794 (8)	235
4-methyl-NAADP (**17**)	42231 ± 28870 (6)	2200
4-*n*-butyl-NAADP (**18**)	> 1000000 (5)	> 50000
4-phenyl-NAADP (**19**)	> 1000000 (6)	> 50000
5-carboxy-NAADP (**22**)	> 1000000 (3)	> 50000

that of NAADP. Compounds **20** and **21** were shown to be approximately equipotent with NAADP, compounds **23** and **24** possessing larger 5-substituents were ca. 10 to 100-fold less potent, while the 4-substituted NAADP derivatives **16** and **17** were from 200- to 2000-fold less potent (Table 3). EC_{50} values for **18**, **19**, and **22** were higher still and could not be accurately determined in this experiment. Compounds **16**, **20**, and **21** elicit maximal calcium release at high concentration and therefore are considered to possess full-agonist activity. The maximal Ca^{2+} release elicited at high concentrations of compounds **23**, **24**, and probably also **17** was shown to plateau at concentrations significantly less than that elicited by NAADP, indicating that these compounds are behaving as partial agonists. Complete concentration−response curves could not be determined for the low potency compounds **18**, **19**, and **22**.

Receptor Desensitization. NAADP and other related agonists characteristically induce desensitization of the Ca^{2+} release mechanism when the receptor is subjected to pretreatment for a few minutes using subthreshold agonist concentrations.[3] Subsequent treatment with a much higher concentration of NAADP is then unable to release calcium. Such desensitization is indicative of involvement of a receptor mediated response and is also a useful tool to demonstrate that an agonist is releasing calcium through the NAADP receptor. It was found that pretreatment with 1 nM 5-CH_3-NAADP (**21**) produced the same characteristic receptor desensitization as

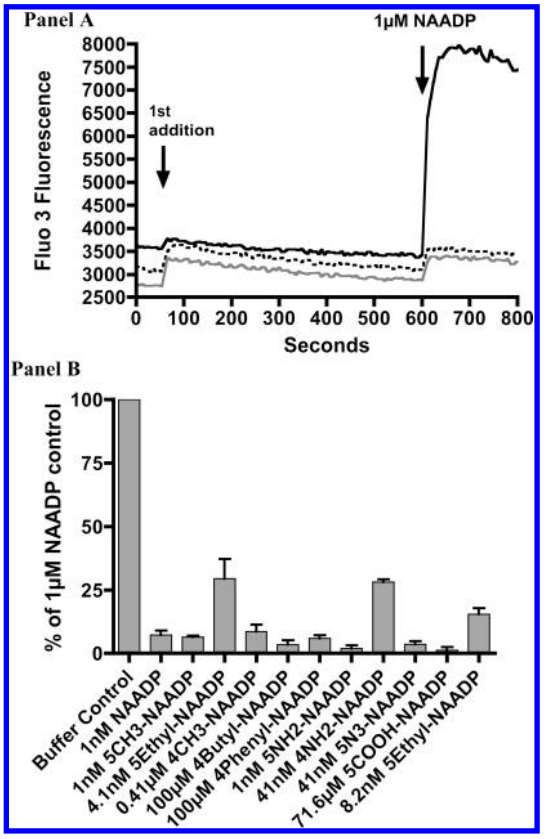


Figure 3. Desensitization of sea urchin egg homogenate Ca^{2+} release by pretreatment with subthreshold concentrations of NAADP or NAADP analogues. (A) Effect of a 9 min pretreatment with buffer (solid line), 1 nM NAADP (**1**) (dotted line), or 1 nM 5-CH_3-NAADP (**21**) (gray line) on calcium release in response to treatment with 1 μM NAADP. The data has been adjusted along the *Y*-axis to prevent overlap. (B) Comparison of the abilities of a 9 min preincubation with NAADP (**1**) or NAADP analogues (**16**−**24**) at the stated concentrations to reduce Ca^{2+} release in response to the addition of 1 μM NAADP. Values shown are mean ± SEM (n = 3).

does NAADP itself (Figure 3A) and hence that **21** must be releasing Ca^{2+} through the same receptor as NAADP itself. Other tested compounds (**16**−**24**) were shown to be similarly effective in desensitization when used for pretreatment at a concentration of 1 nM or higher (Figure 3B). The concentrations of analogues used in Figure 3B were chosen based on previous findings[3,39] that pretreatment with NAADP at subthreshold concentrations (for calcium release) were able to completely desensitize calcium release by a maximal concentration of NAADP. In the experiment shown in Figure 3B, we attempted to use analogue concentrations that were just below the threshold concentration necessary to induce measurable calcium release, approximately 20−100-fold lower than the observed EC_{50}.

Competition Ligand Binding Studies. The ability of NAADP (**1**) and its analogues **16**−**24** to compete with [^{32}P]NAADP for specific binding to sea urchin microsomes was determined using a competition ligand binding assay according to the procedure of Aarhus et al. (1996).[3] The concentration response curves for the 10 tested compounds are shown in Figure 4, and the IC_{50} values derived from the fitted data derived from determinations made in two or more additional experiments in addition to that depicted in Figure 4 are presented in Table 4. The binding studies show that NAADP (**1**), 5-NH_2-NAADP (**20**) and 5-CH_3-NAADP (**21**) inhibit the binding [^{32}P]NAADP by 50%

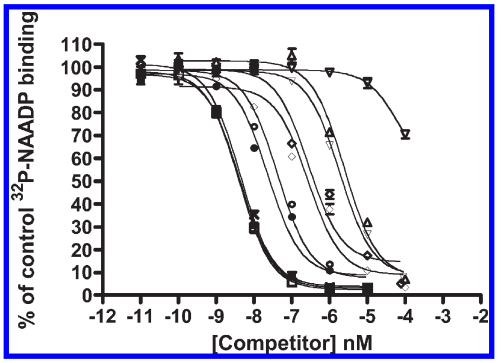


Figure 4. Competition radioligand binding curves for NAADP (**1**) (■) and the nine analogues (**16**−**24**) in sea urchin egg homogenates: 5-NH_3-NAADP (**20**) (□), 5-CH_3-NAADP (**21**) (×), 5-CH_2CH_3-NAADP (**23**) (●), 5-N_3-NAADP (**24**) (○), 4-NH_3-NAADP (**16**) (light ◇), 4-CH_3-NAADP (**17**) (dark ◇), 4-*n*-butyl-NAADP (**18**) (light ▽), 4-phenyl-NAADP (**19**) (dark △), and 5-CO_2H-NAADP (**22**) (dark ▽). Each plotted point represents the mean of a minimum of three determinations.

Table 4. IC_{50} Values Determined for Competition Ligand Binding between [^{32}P]NAADP and NAADP Analogues (**16**−**24**)

compd (structure no.)	IC_{50} ± SD, nM (*n*)	Fold increase in IC_{50}
NAADP (**1**)	1.6 ± 1.5 (9)	1
5-amino-NAADP (**20**)	1.7 ± 1.3 (6)	1.1
5-methyl-NAADP (**21**)	1.9 ± 1.5 (5)	1.2
5-ethyl-NAADP (**23**)	37 ± 30 (4)	23
5-azido-NAADP (**24**)	18 ± 14 (5)	11
4-amino-NAADP (**16**)	141 ± 116 (3)	88
4-methyl-NAADP (**17**)	149 ± 99 (6)	93
4-*n*-butyl-NAADP (**18**)	465 ± 221 (5)	303
4-phenyl-NAADP (**19**)	859 ± 896 (6)	540
5-carboxy-NAADP (**22**)	> 100000 (4)	> 60000

at a concentration range of 1−2 nM, whereas other analogues are able to compete with [^{32}P]NAADP only at much higher concentrations. Of the compounds tested, 5-carboxy-NAADP (**22**) was observed to be the least potent with an EC_{50} of > 100000 nM, > 60000-fold higher than the EC_{50} of NAADP.

Discussion

Although approaches to the chemical synthesis of NAADP and its derivatives have been developed,[40] the process of producing NAADP derivatives synthetically is still lengthy and relatively difficult. Chemoenzymatic syntheses of NAD, NADP, and NAADP derivatives have been effectively used to produce novel pyridine dinucleotides since the discovery of the NAD glycohydrolase catalyzed base exchange reaction in the 1950s. Successful base exchange was found to require a uncharged species, a sufficiently nucleophilic pyridine base ($pK_a > 3$),[41] and usually the absence of sterically interfering substituents *ortho*- to the pyridine nitrogen. A variety of monosubstituted pyridines with large groups in the 3- or 4-positions were found to successfully exchange into the pyridine dinucleotides using the enzyme catalyzed reaction. Disubstituted nicotinamides were studied less often, but 5-methyl-NAD and 5-amino-NAD had been synthesized using the porcine brain NAD-glycohydrolase as a catalyst.[42]

We find that 4-substituted nicotinic acids, even those with large 4-substituents, are excellent exchange substrates. One exception is a cationic compound such as **2a**, which must be converted into an ester before it can be successfully exchanged into the pyridine dinucleotide at a high pH under conditions where the uncharged base is again present at high concentration. We encountered an unexpected limitation of the pyridine base exchange reaction in this study with respect to the synthesis of the 5-substituted NAADP derivatives. Nicotinic acids containing large groups at the 5-position (**3h** or **3j**) or those containing groups which are both electron withdrawing and hydrophobic (**3c**, **3e** (σ_m for acetylene is 0.21 indicating electron withdrawal), or **3f**) fail to exchange. The interesting NAADP derivatives that would be derived from these compounds must therefore be produced by an alternate method. It is interesting that 5-azidonicotinic acid was shown to exchange with the nicotinic acid base of NAADP in good yield to produce **24** (5-azido-NAADP), a potential photoaffinity label for the NAADP binding protein.

When tested as competitive ligands for NAADP binding or for calcium-ion mobilizing activity, we find that NAADP derivatives containing a nicotinic acid moiety with 4-substituents are associated with loss of binding potency. Even small 4-substituents (as in **16** and **17**) are associated with a 100- to 200-fold loss of potency. The 4-substituted compounds are apparently low potency agonists, and we have not yet encountered any 4-substituted NAADP derivatives that behave as antagonists. Subthreshold concentrations of the 4-substituted derivatives **16**−**19** desensitize the NAADP receptor, indicating that these compounds act similarly to the other agonists.

We find that the 5-position of the nicotinic acid ring is tolerant of substitution. Introduction of an amino group (**20**) or a methyl group (**21**) at the 5-position results in the production of derivatives which are only slightly less potent than is NAADP itself. An ethyl group is tolerated with only a 20-fold loss of potency, and the azide (**24**) (a dipolar group containing three linearly disposed heavy atoms) is associated with only a 11-fold increase in IC_{50} as measured in the competition ligand binding assay. The concentration response for Ca^{2+} release induced by 5-ethyl-NAADP (**23**) (Figure 2) apparently plateaus at a significantly lower percent calcium ion release than does the full agonist NAADP, indicating that **23** exhibits partial antagonist activity. Similarly the 5-azide **24** may also be a partial agonist. Most reported modifications of NAADP have been observed to result in the loss of receptor binding potency, and our observation suggests that further modification at the nicotinic acid 5-position might produce potent agonists that will be informative in future studies of receptor function, receptor localization, and receptor isolation. The EC_{50} for 5-azido-NAADP (**24**) is low enough to suggest that when **24** is appropriately labeled, it should prove useful as a photoaffinity label for the identification of NAADP binding proteins (Tables 3 and 4).

The EC_{50} values derived from Ca^{2+} release (Table 3) and the IC_{50} values derived from competition ligand binding (Table 4) are well correlated (see Supporting Information) , but the IC_{50} values are consistently lower. This may be due to the fact that the measurements are made under different experimental conditions. Alternately, it has been suggested recently that the NAADP receptor possesses two binding sites for NAADP, a high affinity inhibitory site and the stimulatory site with a relatively lower affinity.[20] The IC_{50} values measured using the competition ligand binding assay might in such a case reflect the binding potency of the high affinity inhibitory site.

Experimental Section

General Procedures. The following procedures were used in all reactions unless otherwise noted. Oxygen- and moisture-sensitive reactions were carried out in oven-dried ($T > 100$ °C)

glassware sealed under a positive pressure of dry nitrogen supplied from a manifold. Moisture or air sensitive liquids and solutions were transferred under a nitrogen atmosphere by syringe through rubber septa. Reactions were stirred with a Teflon-covered magnetic stirring bar. Reagents for reactions were obtained from commercial sources and were used without further purification, except as indicated. Either sure-seal bottles of anhydrous solvents were purchased from Aldrich or they were purified according to Perrin's Purification of Laboratory Chemicals. Proton (^{1}H) NMR spectra and carbon-13 (^{13}C) NMR spectra were recorded at 600 MHz or at 400 MHz. Chemical shifts are reported in ppm (δ) and are referenced to the residual proton signal of the deuterated solvent. ^{31}P NMR spectra were recorded on Gemini 200 MHz spectrometer with 85% H_3PO_4 as external reference. The pH of the solutions was adjusted to pH 7 prior to the determination of the ^{31}P NMR spectra. The chemical shifts for ^{13}C NMR peaks are reported to the first decimal place, while the peaks which are very close are reported to the second decimal place. The following abbreviations are used to describe spin multiplicity: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, b = broad, dd = doublet of doublets, dt = doublet of triplets, dq = doublet of quartets, and ddd = doublet of doublet of doublets; other combinations derive from those listed. Coupling constants (J) are reported in hertz (Hz). Melting points were determined on an Electrothermal digital melting point apparatus and are uncorrected. Mass spectral analyses were done at University of Toledo Instrumentation Center. Electrospray ionization mass spectra (ESI) were acquired on FT/ICR spectrometer. High resolution mass spectroscopies with accurate mass analyses (HRMS) were performed at Ohio State University Mass Spectrometry and Proteomics facility. Analytical thin layer chromatography (TLC) was performed using 0.25 mm Silica Gel 60 glass plates with a 254 nm fluorescent indicator from Analtech. Plates were developed in a covered chamber and visualized by examination under short wavelength ultraviolet (UV) light, iodine stain, sulfuric acid charring, or ninhydrin stain. Flash chromatography was done using Fisher Silica Gel 60, 200−425 mesh (40−60 mm) as stationary phase. For flash column purification, the amount of stationary phase used was about 40 times the quantity of compound being purified. The silica gel columns were packed in nonpolar solvent, followed by sample application. The column was always washed with 2−3 column volumes of nonpolar solvent to remove highly nonpolar impurities. Solvent evaporation was done under reduced pressure using a Buchi rotavapor either at water aspirator vacuum or at vacuum achieved with a regular vacuum oil pump. Elemental analyses were performed by Atlantic Microlab, Inc. (Norcross, GA), and are regarded as being acceptable when within ±0.4% of the theoretical values.

Anion exchange chromatography was performed using diethylaminoethyl substituted cellulose (DE-52 cellulose from Whatman Inc.). The dimensions of the column used for anion exchange was 1.5 cm × 40 cm. Dowex AG1-X2 resin was obtained in the Cl^- form from Bio-Rad Laboratories (Hercules, CA) was converted from the Cl^- form into acetate form by passing 1 M sodium acetate through the column until the column effluent tested negative for chloride ions. The cation exchange resin, Dowex 50W-X8, was purchased in H^+ form and was converted to tetrabutylammonium form by treating it with an excess of 40% tetrabutylammonium hydroxide.

For base exchange enzymes, 1 unit represents the amount enzyme required to hydrolyze 1 μmol of NAD/min. For the base exchange reaction, the pH of the solution was buffered by the nicotinic acid analogue and was not readjusted after the addition of NADP disodium salt. The catalyst present in base exchange reactions was removed by filtration of the mixture using an Amicon Ultra centrifugal filter device with a molecular weight cut of 10 kDa. The centrifugal filter device was bought from Millipore Corporation (Bedford, MA). The yields of 4- or 5-substituted NAADP derivatives were calculated with respect to NADP.

Materials. 4-Aminonicotinic acid was purchased from AKSci, 5-aminonicotinic acid was bought from Combi-Blocks, Inc., 4-methylnicotinic acid was bought from Maybridge, and 3,5-pyridinedicarboxylate was purchased from Acros Organics. NADP used in pyridine base exchange reactions was bought as the disodium salt in 98% purity from Roche Diagnostics (Indianapolis, IN). (4,5-Dihydro-4,4-dimethyl-2-oxazolyl)pyridine was kindly provided by our colleague Chris Trabbic (University of Toledo).

Synthesis of 4-Substituted Nicotinic Acid Analogues. 4-*n*-Butylnicotinic Acid (2c). The immediate precursor of **2c**, 4-*n*-butyl-3-(4,5-dihydro-4,4-dimethyl-2-oxazolyl)-pyridine, was synthesized by the addition of *n*-butyllithium to the pyridyl-3-oxazoline as follows: 3-(4,5-dihydro-4,4-dimethyl-2-oxazolyl)-pyridine (0.72 g, 4.10 mmol) was taken in a flask and N_2 was flushed through the flask. Anhydrous THF (8.0 mL) was added and the mixture was stirred at room temperature until a solution was formed. The flask was cooled in a −20 °C bath and stirred using a magnetic stirrer. The reaction temperature was regulated using a thermostatically controlled mechanical cryostat. *n*-Butyllithium (1.6 M solution in hexanes, 2.88 mL) was added dropwise over a period of 10 min. The reaction was allowed to stir at −20 °C for 2 h. O_2 gas was bubbled into the reaction for 3 h at 0 °C (bath temperature). The reaction was quenched by adding water (25 mL), and product was extracted into diethylether (3 × 20 mL). The organic layer was dried over sodium sulfate and filtered. The filtrate was concentrated under reduced pressure to an oily residue. This was purified by column chromatography (20−50% ethyl acetate (EtOAc) in hexanes) to obtain product as clear oil (0.67 g, 71%). TLC R_f 0.35 in EtOAc:hexanes (2:3). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.85 (s, 1H), 8.47 (d, 1H, J = 5.2), 7.11 (d, 1H, J = 5.2), 4.05 (s, 2H), 2.94 (t, 2H, J = 8.0), 1.58−1.48 (m, 2H), 1.4−1.3 (m, 2H), 1.36 (s, 6H), 0.89 (t, 3H, J = 7.2).

4-*n*-Butyl-3-(4,5-dihydro-4,4-dimethyl-2-oxazolyl)-pyridine (0.30 g, 1.29 mmol) was dissolved in 3 N HCl (100 mL) and acetic acid (50 mL) and was refluxed at 95 °C for 36 h. The solution was concentrated under reduced pressure and then dissolved in water (20 mL) and neutralized using 2 N NaOH. The aqueous layer was lyophilized ,and the solid was purified by flash chromatography (silica gel, CH_2Cl_2:CH_3OH:acetic acid, 90:9:1) to give **2c** as white solid (0.16 g, 69%): mp 121−123 °C. TLC R_f 0.62 in CH_2Cl_2:CH_3OH:acetic acid (90:9:1). ^{1}H NMR (400 MHz, CD_3OD) δ 8.94 (s, 1H), 8.52 (d, 1H, J = 3.6), 7.40 (d, 1H, J = 3.6), 3.05 (t, 2H, J = 5.2), 1.64−1.58 (m, 2H), 1.46−1.38 (m, 2H), 0.96 (t, 3H, J = 5.2).

4-Phenylnicotinic Acid (2d). The immediate precursor of **2d**, 4-phenyl-3-(4,5-dihydro-4,4-dimethyl-2-oxazolyl)-pyridine, was synthesized by the addition of phenyllithium to pyridyl-3-oxazoline as follows: 3-(4,5-dihydro-4,4-dimethyl-2-oxazolyl)-pyridine (0.57 g, 3.26 mmol) was taken in a flask and N_2 was flushed through the flask. Anhydrous THF (9.5 mL) was added, and the mixture was stirred at room temperature until it formed a solution. The flask was cooled in an acetone/dry ice bath (−78 °C) with stirring. Phenyllithium (2 M solution in ether, 1.6 mL) was added dropwise over a period of 10 min. The reaction was allowed to stir at −78 °C for 1 h and then was stirred at 0 °C for another hour. O_2 gas was bubbled into the reaction for 3 h at 0 °C (bath temperature). The reaction was quenched by adding water (15 mL), and product was extracted in EtOAc (3 × 15 mL). The organic layer was dried over sodium sulfate and filtered. The filtrate was concentrated under reduced pressure to oily residue. This was purified by column chromatography (50% EtOAc in hexanes) to obtain product as colorless oil (612.0 mg, 75%). TLC R_f 0.32 in EtOAc:hexanes (8:7). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.89 (s, 1H), 8.67 (d, 1H, J 5.2), 7.39 (s, 5H), 7.30 (d, 1H, J = 5.2), 3.83 (s, 2H), 1.30 (s, 6H).

4-Phenyl-3-(4,5-dihydro-4,4-dimethyl-2-oxazolyl)-pyridine (0.35 g, 1.38 mmol) was dissolved in 3 N HCl (100 mL) and acetic acid (50 mL) and was refluxed at 95 °C for 36 h. The

solution was concentrated under reduced pressure and then dissolved in water (20 mL) and neutralized using 2 N NaOH. The aqueous layer was lyophilized, and the solid was purified by flash chromatography (CH_2Cl_2:CH_3OH:acetic acid, 90:9:1) to give **2d** as sticky solid (0.20 g, 73%). TLC R_f 0.58 in CH_2Cl_2:CH_3OH:acetic acid (90:9:1). ^{1}H NMR (600 MHz, CD_3OD) δ 8.90 (s, 1H), 8.67 (d, 1H, J = 4.8), 7.49 (d, 1H, J = 4.8), 7.46−7.42 (m, 5H).

Synthesis of 5-Substituted Nicotinic Acid Analogues. 5-Ethynylnicotinic Acid (3e). The immediate precursor of **3e**, 5-ethynyl-3-(4,5-dihydro-4,4-dimethyl-2-oxazolyl)-pyridine, was produced from **6** by removal of the TES-protecting group. Compound **6** (0.56 g, 2.06 mmol) and anhydrous K_2CO_3 (1.46 g, 10.5 mmol) were added to a flask under N_2. Anhydrous CH_3OH (2 mL) and anhydrous THF (2 mL) were added to the flask. The mixture was stirred for 1 h and monitored by TLC. After the reaction was complete, water (15 mL) was added to the flask and product was extracted into EtOAc (2 × 30 mL). The organic layer was dried over sodium sulfate and filtered. The filtrate was concentrated under reduced pressure, and the oily residue was purified by flash chromatography (20−50% EtOAc in hexanes) to obtain 5-ethynyl-3-(4,5-dihydro-4,4-dimethyl-2-oxazolyl)-pyridine as white solid (0.26 mg, 62%); mp 97−98 °C. TLC R_f 0.30 in EtOAc:hexanes (1:1). ^{1}H NMR (600 MHz, CD_3OD) δ 8.99 (d, 1H, J = 1.8), 8.77 (d, 1H, J = 2.4), 8.31 (t, 1H, J = 1.8) 4.26 (s, 2H), 3.92 (s, 1H), 1.39 (s, 6H). ^{13}C NMR (100 MHz, CD_3OD) δ 161.6, 155.5, 149.1, 139.8, 125.2, 121.3, 84.2, 80.8, 80.0, 69.1, 28.5. LC-MS (ESI) m/z 201.2 (M + 1).

5-Ethynyl-3-(4,5-dihydro-4,4-dimethyl-2-oxazolyl)-pyridine (0.8 g, 4 mmol) was dissolved in 3 N HCl (150 mL) and acetic acid (75 mL), and the mixture was refluxed at 95 °C for 36 h. The solution was concentrated under reduced pressure and then dissolved in water (25 mL) and neutralized using 2 N NaOH. The aqueous layer was lyophilized, and the solid was purified by flash chromatography to give **3e** as white solid (0.43 g, 74%); mp 210−211 °C with decomposition. TLC R_f 0.60 in CH_2Cl_2:CH_3OH:acetic acid (90:9:1). ^{1}H NMR (400 MHz, CD_3OD) δ 9.00 (d, 1H, J = 1.6), 8.62 (d, 1H, J = 2.0), 8.34 (t, 1H, J = 2.0), 3.79 (s, 1H). ^{13}C NMR (100 MHz, CD_3OD) δ 167.0, 156.4, 150.7, 141.6, 128.3, 121.4, 84.0, 80.0. Analysis Calcd for $C_8H_5NO_2$ 0.1 H_2O: C 64.52, H 3.52, N 9.40. Found: C 64.29, H 3.33, N 9.31. LC-MS (ESI) m/z 148.3 (M + 1).

5-Ethenylnicotinic Acid (3f). To a cooled solution of 5-ethynyl nicotinic acid (**3e**) (0.1 g, 0.68 mmol) in CH_3OH (10 mL) were added Lindlar's catalyst (5 mg, 5%) and 2,2′-(ethylenedithio)diethanol (50 μg, 10 parts per thousand of Pd catalyst). The mixture was shaken under H_2 atmosphere (30 psi) at room temperature. The reaction was monitored by TLC and was filtered through a Celite pad after 24 h. The filtrate was concentrated under reduced pressure at room temperature, and the crude product was purified by column chromatography in CH_2Cl_2:CH_3OH:acetic acid (90:9:1) to give **3f** (27.4 mg, 27%); mp 154−156 °C. TLC R_f 0.55 in CH_2Cl_2:CH_3OH:acetic acid (90:9:1). ^{1}H NMR (600 MHz, CD_3OD) δ 8.98 (s, 1H), 8.76 (s, 1H), 8.45 (s, 1H), 6.87−6.82 (dd, 1H, ^2J = 18, ^3J = 11.4), 6.02 (d, 1H, J = 17.4), 5.50 (d, 1H, J = 10.8). ^{13}C NMR (150 MHz, CD_3OD) δ 166.6, 150.5, 148.9, 134.3, 133.9, 132.4, 127.5, 117.3. LC-MS (ESI) m/z 150.1 (M + 1).

5-Ethylnicotinic Acid (3g). In a flask under N_2, 5-ethynylnicotinic acid (**3e**) (0.04 g, 0.3 mmol) and ammonium formate (0.063 g, 1 mmol) were added. To this flask, a mixture of 10% Pd−C (50 mg) and cold CH_3OH (5 mL) was added and the resulting mixture was stirred for 12 h. The Pd−C was filtered off through a bed of Celite, and the clear filtrate was evaporated. The residue was washed with methylene chloride several times, and the product went into the methylene chloride. The solvent was evaporated to give product **3g** as sticky residue (25 mg, 78%). ^{1}H NMR (600 MHz, CD_3OD) δ 8.93 (s, 1H), 8.57 (s, 1H), 8.25 (s, 1H), 2.76 (q, 2H, ^2J = 15.0, ^3J = 7.2), 1.29 (t, 3H, J = 7.2). ^{13}C NMR (150 MHz, CD_3OD) δ 167.6, 151.6, 147.4, 140.3, 137.2, 128.4, 25.5, 14.4. LC-MS (ESI) m/z 152.1 (M + 1).

5-Phenylnicotinic Acid (3h). 5-Phenyl-3-(4,5-dihydro-4,4-dimethyl-2-oxazolyl)-pyridine (**9**) (0.35 g, 1.38 mmol) was dissolved in 3 N HCl (100 mL) and acetic acid (50 mL) and was refluxed at 95 °C for 36 h. The solution was concentrated under reduced pressure and then dissolved in water (20 mL) and neutralized using 2 N NaOH. The aqueous layer was lyophilized, and the solid was purified by flash chromatography (CH_2Cl_2:CH_3OH:acetic acid, 90:9:1) to give product as white solid (215 mg, 78%); mp 256−257 °C (reported mp 267−269 °C[44]). TLC R_f 0.58 in CH_2Cl_2:CH_3OH:acetic acid (90:9:1). ^{1}H NMR (600 MHz, CD_3OD) δ 9.10 (d, 1H, J = 1.8), 9.00 (d, 1H, J = 2.4), 8.60 (t, 1H, J = 1.8), 7.73 (d, 2H, J = 7.2), 7.55−7.45 (m, 3H). ^{13}C NMR (100 MHz, CD_3OD) δ 150.6, 148.6, 137.3, 136.5, 135.8, 129.2, 128.7, 128.0, 127.7, 127.0.

5-Azidonicotinic Acid (3i). 5-Azidonicotinic acid ethyl ester (**11**) (2.5 g, 13.02 mmol) was dissolved in CH_3OH (15 mL). The solution was stirred at room temperature for 10 min, and 2 N NaOH (10 mL) was added to it. This solution was then stirred at room temperature, and the reaction was monitored by TLC for disappearance of starting material (24 h). The solution was concentrated under reduced pressure and was diluted with water (15 mL). The basic layer was then carefully neutralized with 2 N HCl, resulting in precipitation of white solid. The solid was filtered and dried under vacuum to give product (1.69 g, 79%); mp 178−180 °C. TLC R_f 0.53 in CH_2Cl_2:CH_3OH:acetic acid (90:9:1). ^{1}H NMR (600 MHz, CD_3OD) δ 8.88 (d, 1H, J = 1.8), 8.48 (d, 1H, J = 2.4), 8.05 (t, 1H, J = 1.8).; ^{13}C NMR (150 MHz, CD_3OD) δ 167.1, 147.4 (d, J = 6.9), 145.4 (d, J = 6.15) 139.6, 129.4, 128.5. Analysis Calcd for $C_6H_4N_4O_2$: C 43.91, H 2.46, N 34.14. Found: C 44.00, H 2.4, N 33.88. LC-MS (ESI) m/z 165.2 (M + 1).

5-(4-(Aminomethyl)-1*H*-1,2,3,-triazol-1-yl)nicotinic Acid (3j). A solution of ethyl 5-(4-(aminomethyl)-1*H*-1,2,3-triazol-1-yl)nicotinate (**14**) (100 mg, 0.4 mmol) in CH_3OH (2 mL) and NaOH (2 M, 0.5 mL, 1 mmol) was stirred for 24 h at room temperature. When no starting material was detected on TLC (CH_2Cl_2:CH_3OH:TEA, 8:1:1), the solution was neutralized with 2 N HCl and concentrated in vacuum. The residue was dissolved in water (10 mL) and lyophilized. The solid obtained was then suspended in CH_3OH and filtered. The filtrate was then concentrated in vacuum to obtain **3j** as white solid with some inorganic salts (85 mg). ^{1}H NMR (400 MHz, CD_3OD) δ 9.27 (s, 1H), 9.23 (s, 1H), 8.83 (s, 1H), 8.81 (t, 1H), 4.39 (s, 1H). ^{13}C NMR (150 MHz, CD_3OD) δ 163.1, 151.7, 141.4, 130.4, 124.4, 119.8, 116.9, 35.5.

3-Bromo-5-(4,5-dihydro-4,4-dimethyl-2-oxazolyl)-pyridine (4). Compound **4** was prepared from **3c** in three steps according to the procedure of Robert et al., (2006)[43] and isolated as a white solid (4.92 g, 78% over three steps); mp 91−93 °C (lit.[43] 90−91 °C). TLC R_f 0.65 in EtOAc. The NMR data was in agreement with the published data.

1,2-Bis(5-(4,4-dimethyl-4,5-dihydrooxazol-2y)pyridine-3-yl)-ethyne (5). To a two-neck flask, triethyl(ethynyl)silane (TES-acetylene, 182 mg, 1.3 mmol) was added and it was protected from air under N_2 atmosphere. To this flask, a solution of tetrakis-(triphenylphosphine)-palladium(0) (45 mg, 0.04 mmol) in ethanol/dimethoxyethane (1:1, 2 mL), aqueous sodium carbonate (2 M, 4 mL), and copper iodide (46 mg, 0.24 mmol) was added. The resultant solution was stirred at room temperature for 15 min, and then to this solution 3-bromo-5-(4,5-dihydro-4,4-dimethyl-2-oxazolyl)-pyridine (**4**) (0.6 g, 2.5 mmol) was added under a stream of nitrogen. The flask was then stirred at 90 °C (oil bath temperature) for 1 h, and the reaction was brought to room temperature. The mixture was poured into a flask containing anhydrous sodium sulfate, filtered, and evaporated in vacuum. The residue was then purified by column chromatography (10−50% EtOAc in hexanes) to give compound **5** (155 mg, 58%). TLC R_f 0.30 in EtOAc:hexanes (1:1). ^{1}H NMR (600 MHz, $CDCl_3$) δ 9.05 (s, 1H), 8.79 (s, 1H), 8.32 (t, 1H, J = 1.8), 4.12 (s, 2H), 1.36 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 159.6, 154.0, 148.8, 138.7, 138.0, 124.1, 119.6, 89.2, 68.2, 28.6. LC-MS (ESI) m/z 375.5 (M + 1).

4,4-Dimethyl-2-(5-((triethylsilyl)ethynyl)pyridine-3-yl)-4,5-dihydrooxazole (6). A two-neck flask, under N_2, was charged with 3-bromo-5-(4,5-dihydro-4,4-dimethyl-2-oxazolyl)-pyridine (**4**) (1.6 g, 6.27 mmol) and benzyltributyl ammonium bromide (1.8 g, 5.05 mmol). To this mixture, degassed acetonitrile (9 mL) and degassed water (0.9 mL) were added. This was followed by addition of TES-acetylene (1.4 mL, 7.7 mmol) and DIPEA (7.2 mL). The mixture was stirred well at ambient temperature. In another flask, $Pd(OAc)_2$ (222.5 mg, 1 mmol) and PPh_3 (525 mg, 2 mmol) were taken and dissolved in degassed acetonitrile (45 mL) and water (4.5 mL). The latter was added to the two-neck flask and was stirred at ambient temperature for 8 h. The reaction was monitored by TLC, and when the starting material had disappeared, water (20 mL) was added to quench the reaction. Product was extracted into EtOAc (3 × 30 mL), and the organic layer was dried over sodium sulfate and filtered. The filtrate was concentrated under reduced pressure, and the oily residue was purified by flash chromatography (20−50% EtOAc in hexanes). Product was obtained as brown oil (1.16 g, 68%) that solidified on freezing; mp 67−69 °C. TLC R_f 0.54 in EtOAc:hexanes (1:1). ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.96 (s, 1H), 8.70 (s, 1H), 8.25 (t, 1H, J = 1.8), 4.09 (s, 2H), 1.34 (s, 6H), 0.99 (t, 9H, J = 8.4), 0.63 (q, 6H, ^2J = 16.2, ^3J = 7.8). ^{13}C NMR (150 MHz, $CDCl_3$) δ 159.6, 154.4, 147. 9, 138.4, 123.5, 120.2, 101.6, 96.8, 79.3, 67.9, 28.3, 7.42 (d, J = 1.95), 4.18. LC-MS (ESI) m/z 315.3 (M + 1).

5-(4,5-Dihydro-4,4-dimethyl-2-oxazolyl)-3-pyridinylboronic Acid (7). 3-Bromo-5-(4,5-dihydro-4,4-dimethyl-2-oxazolyl)-pyridine (**4**) (1.5 g, 5.90 mmol) was taken in a flask and flushed with N_2. Anhydrous toluene (5 mL) and anhydrous THF (6.5 mL) were added to the flask, and the mixture was stirred until the solid dissolved. The flask was then transferred to a −40 °C cooling bath and stirred rapidly. Some of the solid started to precipitate. Triisopropyl borate (1.8 mL, 7.8 mmol) was added to the flask. When the reaction mixture had been cooled for 15 min, *n*-butyllithium (1.6 M in hexanes, 4.5 mL, 7.2 mmol) was added dropwise over a period of 1 h. By the time butyllithium addition was complete, the mixture turned into a yellowish viscous liquid. It was stirred for another 30 min at −40 °C, brought to −20 °C, and 2 N HCl (20 mL) added. The acidic layer was extracted with toluene (20 mL), the toluene extract was discarded, and then the aqueous layer was neutralized with 2 N NaOH. The product was extracted into THF (4 × 15 mL). The THF layer was evaporated to dryness, and the residue was dissolved in mixture of CH_3OH/THF (1:1) and filtered. The filtrate was evaporated under reduced pressure and the residue crystallized from acetonitrile to obtain boronic acid **7** as yellow solid (0.69 g, 53%); mp 270−272 °C with decomposition.

Pinacol Ester of 5-(4,5-Dihydro-4,4-dimethyl-2-oxazolyl)-3-pyridinyl Boronic Acid (Pinacol Ester 8). 5-(4,5-Dihydro-4,4-dimethyl-2-oxazolyl)-3-pyridinylboronic acid (**7**) (0.1 g, 0.4 mmol), $(CH_3)_2COHCOH(CH_3)_2$, (190 mg, 1.6 mmol), and toluene (4 mL) were taken in a two-neck flask equipped with N_2 inlet and condenser with a Dean−Stark trap. The mixture was heated in a 120 °C oil bath and refluxed for 2.5 h. The reaction was monitored with TLC, and upon completion the solution was concentrated in vacuum. The residue was purified using column chromatography (10−50% EtOAc in hexanes) to obtain product as yellow oil (63.2 mg, 46%). TLC R_f 0.30 in EtOAc:hexanes (1:1). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.14(s, 1H,), 8.97 (s, 1H,), 8.59 (d, 1H, J = 1.6) 4.10 (d, 2H, J = 1.6), 1.36 (d, 6H, J = 1.2), 1.32 (d, 12 H, J = 1.2). LC-MS (ESI) m/z 303.3 (M + 1).

5-Phenyl-3-(4,5-dihydro-4,4-dimethyl-2-oxazolyl)-pyridine (9). A two-neck flask fitted with a N_2 inlet was charged with tetrakis-(triphenylphosphine)palladium(0) (230 mg, 0.2 mmol). Degassed dimethoxyethane (6 mL) and degassed sodium carbonate (2 M, 4 mL) were added to the flask. Bromobenzene (0.45 mL, 4.2 mmol) was added to the mixture, and the suspension was stirred at room temperature for 15 min. In a separate flask, 5-(4,5-dihydro-4,4-dimethyl-2-oxazolyl)-3-pyridinyl boronic acid (**7**) (0.7 g, 3.18 mmol) was dissolved in degassed EtOH (6 mL) and added to the catalyst mixture. The mixture was allowed to stir at room temperature for 15 min. The flask was sealed with a rubber septum and transferred to oil bath at 90 °C to stir for 4 h. The reaction was monitored by TLC, and when almost all starting material disappeared, the reaction mixture was filtered through Celite, washing with CH_2Cl_2. The organic layer was dried over sodium sulfate and filtered. The filtrate was concentrated in vacuum, and the residue was purified by column chromatography (40−70% EtOAc in hexanes) to obtain **9** as oil (490 mg, 61%). TLC R_f 0.55 in EtOAc:hexanes (7:3). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.07 (d, 1H, J = 2.0), 8.89 (d, 1H, J = 2.4), 8.39 (t, 1H, J = 2.4), 7.6 (d, 2H, J = 7.2), 7.46−7.37 (m, 3H) 4.13 (s, 2H), 1.38 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 160.4, 150.5, 148.2, 134.1, 129.3, 128.7, 127.5, 79.5, 68.2, 28.6.

5-Aminonicotinic Acid Ethyl Ester (10). 5-Aminonicotinic acid (**3a**) (5.0 g, 0.036 mol) was dissolved in EtOH (200 mL) and conc HCl (40 mL). The solution was then refluxed for 36 h. The reaction was monitored by TLC and was concentrated under reduced pressure upon completion. The resulting residue was dissolved in distilled water (25 mL) and neutralized using 1 N NaOH. The aqueous layer was then extracted with EtOAc (3 × 50 mL). The organic layer was dried over sodium sulfate and filtered. The filtrate was concentrated under reduced pressure to obtain white crystalline solid. The solid was dried under vacuum to obtain **10** (4.9 g, 82%); mp 95−97 °C. TLC R_f 0.66 in CH_3OH:CH_2Cl_2 (1:9). ^{1}H NMR (600 MHz, CD_3OD) δ 8.32 (d, 1H, J = 1.8), 8.10 (d, 1H, J = 2.4), 7.59−7.60 (m, 1H), 4.36 (q, 2H, ^2J = 14.4, ^3J = 7.2), 1.38 (t, 3H, J = 7.2). ^{13}C NMR (150 MHz, CD_3OD) δ 167.0, 146.8, 140.6, 138.8, 128.4, 122.6 (d, J = 8.25) 62.56, 14.7. Analysis calcd for $C_8H_{10}N_2O_2$: C 57.82, H 6.07, N 16.86. Found: C 57.80, H 6.17, N 16.67.

5-Azidonicotinic Acid Ethyl Ester (11). 5-Aminonicotinic acid ethyl ester (**10**) (3.0 g, 18.05 mmol) was stirred with concentrated HCl (100 mL) in a 0 °C bath. A solution of $NaNO_2$ (1.38 g, 19.86 mmol) in ice water (8 mL) was added dropwise to the stirred slurry of the hydrochloride salt, resulting in a clear solution. This solution was then added to stirred slurry of NaN_3 (1.77 g, 27.09 mmol) and sodium acetate (3.9 g) in ice water (3.5 mL). After a few minutes of stirring, a red oil separated from the mixture. The mixture was made basic by the addition of concentrated ammonium hydroxide solution after 10 min, and the product was extracted into EtOAc. The organic layer was dried over sodium sulfate, filtered, and the filtrate concentrated under reduced pressure. The residue was purified by column chromatography (10% EtOAc in hexanes) to give **11** as golden oil[34] (1.66 g, 48%). TLC R_f 0.48 in EtOAc:hexanes (1:5). ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.96 (d, 1H, J = 1.6), 8.47 (d, 1H, J = 2.8), 7.94 (d, 1H, J = 2.0), 4.41 (q, 2H, ^2J = 14.4, ^3J = 7.2), 1.40 (t, 3H, J = 7.2). ^{13}C NMR (100 MHz, $CDCl_3$) δ 164.7, 147.0, 144.9, 137.5, 127.2, 126.7, 62.1, 14.4. Analysis calcd for $C_8H_8N_4O_2$ 0.1 EtOAc: C 50.20, H 4.41, N 27.88. Found: C 49.82, H 4.21, N 27.92.

Ethyl 5-(4-((*tert*-Butyloxycarbonylamino)methyl)-1*H*-1,2,3-triazol-1-yl)nicotinate (13). To a solution of 5-azidonicotinic acid ethyl ester (**11**) (150 mg, 0.78 mmol) and *tert*-butyl prop-2-ynylcarbamate (**12**) (124 mg, 0.8 mmol) in THF (1 mL) were added CuI (300 mg, 1.6 mmol) and DIPEA (0.4 mL, 2.4 mmol). The reaction was stirred at room temperature for 12 h, by which time all the starting material was consumed. The reaction mixture was diluted with water (10 mL) and saturated aqueous NH_4Cl (10 mL) and was extracted with CH_2Cl_2 (3 × 20 mL). The organic layers were combined and washed with brine, dried over anhydrous sodium sulfate, and filtered. The filtrate was concentrated under reduced pressure, and the residue was purified by column chromatography to obtain impure material (160 mg), which was used for next step without further purification.

Ethyl 5-(4-(Aminomethyl)-1*H*-1,2,3-triazol-1-yl)nicotinate (14). To a solution of **13** (160 mg) in anhydrous CH_2Cl_2 (2 mL) was added triflouroacetic acid (2 mL), and it was stirred at room temperature for 1.5 h. The reaction was concentrated in vacuum

and again diluted with CH_2Cl_2 (5 mL). This solution was then neutralized with triethylamine (TEA) and concentrated in vacuum. The residue was purified using column chromatography (CH_2Cl_2:CH_3OH:TEA, 8:1:1) to give **14** as colorless oil (92.6 mg, 48% over two steps with respect to 5-azidonicotinic acid ethyl ester). TLC R_f 0.65 in CH_2Cl_2:CH_3OH:TEA (8:1:1). ^{1}H NMR (400 MHz, CD_3OD) δ 9.30 (d, 1H, J = 2.4), 9.22 (d, 1H, J = 1.6), 8.82 (s, 1H), 8.79 (t, 1H, J = 2.0), 4.47 (q, 2H, ^2J = 14.4, ^3J = 7.2), 4.39 (s, 1H), 1.44 (t, 3H, J = 7.2). ^{13}C NMR (100 MHz, CD_3OD) δ 165.3, 151.3, 146.1, 142.9, 135.1, 130.0, 129.0, 124.3, 63.4, 35.5, 14.6. LC-MS (ESI) m/z 248 (M + 1).

Synthesis of NAADP Analogues. Enzymatic Assay of NAD Glycohydrolase Activity.[45] Recombinant *Aplysia* ADP-ribosyl cyclase expressed in yeast was produced according to procedure of Lee et al.[46] NAD glycohydrolase (NADase) from pig brain was bought from Sigma-Aldrich as an acetone powder. The dry acetone powder was hydrated and dispersed into a fine suspension each time immediately before use. The stock mixture consisted of 60 mg of dry acetone powder in 2 mL of distilled water. The mixture was kept at 37 °C for 0.5 h and then subjected to homogenization until fine milky suspension was obtained. The NADase from beef spleen was isolated according to procedure of Kaplan and Colowick.[47] The NADase activity of *Aplysia* cyclase, NADase (pig brain), and NADase (beef spleen) were measured by determining the amount of NAD present at timed intervals in the assay by the reduction of NAD to NADH catalyzed using yeast alcohol dehydrogenase. The NAD glycohydrolase test solution was made by addition of sodium dihydrogen phosphate pH 7.4 (0.1 M, 0.8 mL), NADase enzyme (80 μL of stock made up to 0.2 mL with buffer), and NAD (0.2 mL, 6 mg/mL of buffer, 1.808 μmol). The reaction was incubated at 37 °C for 10 min and trichloroacetic acid (TCA) (25% w/v, 0.3 mL) was added to quench the reaction. The blank solution was treated exactly the same way, except that TCA was added even before initiating the reaction. Both the test and the blank solutions were centrifuged at the speed of 3200*g* for 5 min, and 0.3 mL of aliquot was removed from the clear supernatant. This was added to a cuvette with Tris-ethanol buffer pH 10 (2.7 mL, equal volumes of 0.5 M Tris and 0.5 M ethyl alcohol). To this solution, yeast alcohol dehydrogenase was added (10 μL, 10 mg/mL) and the OD was measured at 340 nm.

Enzymatic Assay for NADP Estimation.[48] NADP, glucose-6-phosphate, and yeast glucose-6-phosphate dehydrogenase (2 units in 10 mL solution) were obtained commercially. In the chemoenzymatic synthesis of NAADP analogues, NADP was incubated with huge excess of nicotinic acid in the presence of NAD glycohydrolase. The reaction was monitored by determination of the amount of NADP left in the reaction mixture. A 20 μL aliquot was removed from the reaction mixture (with concentration of about 0.01 mmol of NADP/mL) at intervals of 0.5 h and the amount of NADP determined until complete disappearance of NADP was observed. For the determination, 2-(*N*-morpholino)ethanesulfonic acid buffer (MES) (50 mM, 1 mL) was taken in a cuvette. To this the reaction aliquot and glucose-6-phosphate (0.1 M, 40 μL) were added. The NADP present in the reaction mixture is converted into NADPH by addition of 10 μL of a solution (5 mg/mL of enzyme) of yeast glucose-6-phosphate dehydrogenase at room temperature. The NADPH that was formed showed absorbance at 340 nm, and the time when no absorbance was seen in the assay was indicative of reaction completetion. This is a very sensitive assay which is capable of detecting nM concentrations of NADP. At the time at which the starting NADP concentration was determined to be zero, all the NADP was either exchanged into NAADP or was converted into ADPRP as a result of a competing hydrolyzing reaction.

Characterization of Pyridine Dinucleotide Analogues 16−24. All compounds were examined by high pressure liquid chromatography (HPLC) using AG MP-1 chromatography[49] immediately prior to testing and if necessary repurified by preparative HPLC using this same system before analysis. HPLC traces are shown for all tested compounds in the Supporting Information. This chromatographic system was capable of separating the pyridine dinucleotide precursor, the cyclic ADP-ribose derivative, and the linear ADP-ribose derivative in each case. Pyridine dinucleotides were also judged pure by determining that the aromatic and anomeric regions of the ^{1}H NMR spectrum were free from extraneous signals and that the ^{31}P NMR exhibited no unexplained signals besides the expected phosphate monoester and the pyrophosphoryl resonance.

4-Amino-NAADP Methyl Ester (15). The requisite pyridine base, 4-aminonicotinic acid methyl ester, was produced by esterification of the acid **2a**. A solution of 4-aminonicotinic acid (**2a**) (200 mg, 1.45 mmol) in anhydrous methanol (25 mL) and conc HCl (3 mL) was refluxed for 24 h with constant stirring. The condenser was fitted with a Drierite trap at the top. When the starting material had converted to a nonpolar material, as shown by TLC, all the volatiles were removed in vacuum. The residue was again dissolved in distilled water and was neutralized with NaOH (1 M). The aqueous layer was then extracted with EtOAc (3 × 20 mL). All the organic extracts were combined, dried over anhydrous sodium sulfate, and evaporated to give white crystalline material (92.5 mg, 42%); mp 172−174 °C. TLC R_f 0.65 in CH_3OH:CH_2Cl_2 (1:9). ^{1}H NMR (600 MHz, CD_3OD) δ 8.67 (s, 1H), 8.00 (d, 1H, J = 6.0), 6.68 (d, 1H, J = 6.0), 3.88 (s, 3H). ^{13}C NMR (150 MHz, CD_3OD) δ 167.6, 156.4, 151.9, 150.2, 110.9, 107.0, 51.0.

A mixture of 4-aminonicotinic acid methyl ester (30 mg, 0.19 mmol) was made in distilled water (1.5 mL) and tris-(hydroxymethyl)aminomethane (Tris) HCl buffer at pH 7.5 (10 mM, 1.5 mL). The pH of the mixture was readjusted to 7.5 with Tris base (0.5 M) and the mixture turned to solution on stirring at 37 °C. To this solution, NADP (13 mg, 0.017 mmol) and *Aplysia* cyclase (60 μL, 0.25 U) enzyme were added. The solution was stirred for 4.5 h at 37 °C. The reaction was monitored by an assay to determine the amount of NADP left in the reaction. Upon completion, the catalyst was removed from the reaction solution by centrifuging in Amicon Ultra centrifugal filter device (10000 molecular weight cut) at the speed of 3200*g* for 15 min. The filtrate contained the product, and the retentate was discarded. The clear filtrate was diluted, adjusted to pH 7.0, and purified by anion exchange chromatography on DEAE 52 cellulose. A gradient of 10−600 mM NH_4HCO_3 (700 mL) was applied and the product eluted at around 290 mM NH_4HCO_3. The appropriate fractions were lyophilized to obtain product as white solid (6 mg, 44%). ^{1}H NMR: 8.70 (s, 1H), 8.25 (s, 1H), 8.08 (s, 1H), 8.06 (d, 1H, J = 7.2), 6.79(d, 1H, J = 7.2), 6.09 (d, 1H, J = 5.4), 5.57 (d, 1H, J = 6.0), 4.95 (m, 1H), 4.18−4.60 (9H), 3.89 (s, 3H). ^{31}P NMR: 0.56, −11.20.

4-Amino-NAADP (16). 4-Amino-NAADP methyl ester (**15**) (6 mg, 0.008 mmol) was dissolved in 3 mL of a 0.2 M solution of TEA dissolved in H_2O/CH_3OH. The solution was stirred at 35 °C for precisely 12 h. The reaction mixture was diluted with distilled water, and CO_2 was bubbled through it until the pH went down to 7.0. This was followed by lyophilization to give white solid with some triethyl ammonium bicarbonate buffer. Repeated lyophilizations gave the product as tris(triethylammonium) salt in quantitative yields. ^{1}H NMR: 8.37 (s, 1H), 8.34 (s, 1H), 8.08 (s, 1H), 7.88 (d, 1H, J = 7.2), 6.63 (d, 1H, J = 7.2), 6.13 (d, 1H, J = 5.4), 5.52 (d, 1H, J = 6.0), 5.05 (m, 1H), 4.6 (m, 1H), 4.15−4.40 (8H). ^{31}P NMR: 2.42, −10.59.

4-Methyl-NAADP (17). A solution of 4-methylnicotinic acid HCl salt (**2b**·HCl, 70 mg, 0.51 mmol) was made in distilled water (4 mL). The pH of the mixture was adjusted to 4.0 with 0.5 M NaOH and a solution resulted. Then 2.5 mL of this solution was added to another vial, followed by solid NADP (20 mg, 0.027 mmol) and *Aplysia* cyclase enzyme (60 μL, 0.25 U). The pH of the solution was not readjusted and was buffered by the nicotinate solution. The reaction was stirred for 2.5 h at 37 °C. The reaction was monitored by an enzymatic assay to determine the amount of NADP left in the reaction. Upon completion, the

catalyst was removed from the reaction solution by centrifuging in an Amicon Ultra centrifugal filter device (10000 molecular weight cut) at the speed of 3200*g* for 15 min. The filtrate contained the product, and the retentate was discarded. The clear filtrate was diluted, adjusted to pH 7.0, and purified by anion exchange chromatography on DE-52 cellulose. A gradient of 10−600 mM NH_4HCO_3 (700 mL) was applied and the product eluted at around 300 mM NH_4HCO_3. The appropriate fractions were lyophilized to obtain product as white solid (8.8 mg, 43%). ^{1}H NMR: 8.73 (s, 1H), 8.63 (d, 1H, J = 6.6), 8.41 (s, 1H), 8.08 (s, 1H), 7.76 (s, 1H, J = 6.0), 6.14 (d, 1H, J = 6.0), 5.84 (d, 1H, J = 5.4), 5.10 (m, 1H), 4.63−4.15 (9H), 2.60 (s, 3H). ^{31}P NMR: 0.66, −10.46. HRMS: 781.0575 (M + Na).

4-*n*-Butyl-NAADP (18). A solution of 4-butylnicotinic acid (**2c**, 30 mg, 0.17 mmol) was made in distilled water (2.5 mL). The pH of the solution was adjusted to 4.0 with 0.5 M NaOH. Then this solution was added to another vial, followed by addition of NADP (14 mg, 0.02 mmol) and *Aplysia* cyclase enzyme (60 μL, 0.25 U). The solution was buffered by the nicotinate solution. The reaction was stirred for 1 h at 37 °C. The reaction was monitored by an assay to determine the amount of NADP left in the reaction. Upon completion, the catalyst was removed from the reaction solution by centrifuging in an Amicon Ultra centrifugal filter device (10000 molecular weight cut) at the speed of 3200*g* for 15 min. The filtrate contained the product and the retentate was discarded. The clear filtrate was diluted, adjusted to pH 7.0, and purified by anion exchange chromatography on DE-52 cellulose. A gradient of 10−600 mM NH_4HCO_3 (700 mL) was applied and the product eluted at around 360 mM NH_4HCO_3. The appropriate fractions were lyophilized to obtain product as white solid (9 mg, 60%). ^{1}H NMR: 8.69 (s, 1H), 8.66 (d, 1H, J = 6.4), 8.41 (s, 1H), 8.09 (s, 1H), 7.79 (d, 1H, J = 6.4), 6.14 (d, 1H, J = 5.6), 5.84 (d, 1H, J = 5.2), 5.10 (m, 1H), 4.62−4.10 (9H), 2.93 (m, 2H), 1.51 (m, 2H), 1.26 (m, 2H), 0.84 (t, 3H, J = 7.6). ^{31}P NMR: 0.66, −10.46. HRMS: 801.1229 (M + Na).

4-Phenyl-NAADP (19). A solution of 4-phenylnicotinic acid (**2d**, 30 mg, 0.15 mmol) was made in distilled water (2.5 mL). The pH of the solution was adjusted to 4.0 with 0.5 M KOH solution. Then this solution was added to another vial, followed by addition of solid NADP (14 mg, 0.02 mmol) and *Aplysia* cyclase enzyme (60 μL, 0.25 U). The solution was stirred for 1 h at 37 °C. The reaction was monitored by an assay to determine the amount of NADP left in the reaction. Upon completion the reaction mixture was centrifuged and filtered in centrifugal filter tubes at 3200*g* for 15 min. The clear filtrate was diluted, adjusted to pH 7.0, and then applied to DEAE 52 cellulose anion exchange purification. A gradient of 10−600 mM NH_4HCO_3 (700 mL) was applied and the product eluted at around 400 mM NH_4HCO_3. The appropriate fractions were lyophilized to obtain product as white solid (8 mg, 51%). ^{1}H NMR: 8.78 (d, 1H, J = 6.4), 8.68 (s, 1H), 8.19 (s, 1H), 7.87 (s, 1H), 7.77 (d, 1H, J = 6.4), 7.34−7.26 (m, 5 H) 5.86 (t, 2H, J = 4.8), 5.9−5.8 (m, 1H), 4.43−4.00 (9H). ^{31}P NMR: 0.51, −10.62. HRMS: 821.0932 (M + Na).

5-Amino-NAADP (20). A mixture of 5-aminonicotinic acid (**3a**, 69 mg, 0.50 mmol) was made in distilled water (6 mL). The mixture was stirred at 37 °C for 1 h, but some of the solid did not dissolve. The clear supernatant (3 mL) was taken and the pH was adjusted to 4.0 with 0.5 M KOH. To this solution, solid NADP (16 mg, 0.021 mmol) was added, followed by *Aplysia* cyclase enzyme (80 μL, 0.33 U). The reaction was buffered by the nicotinate solution. The reaction was stirred for 3.5 h at 37 °C. The reaction was monitored by an assay to determine the amount of NADP left in the reaction. Upon completion, the catalyst was removed from the reaction solution by centrifuging in an Amicon Ultra centrifugal filter device (10000 molecular weight cut) at the speed of 3200*g* for 15 min. The filtrate contained the product, and the retentate was discarded. The clear filtrate was diluted, adjusted to pH 7.0, and purified by anion exchange chromatography on DE-52 cellulose. A gradient of 10−600 mM NH_4HCO_3 (700 mL) was applied and the product eluted at around 330 mM NH_4HCO_3. The appropriate fractions were lyophilized to obtain product as white solid (7 mg, 43%). ^{1}H NMR: 8.40 (s, 1H), 8.27 (s, 1H), 8.20 (s, 1H), 8.13 (s, 1H), 7.77 (s, 1H), 6.14 (d, 1H, J = 4.8), 5.80 (d, 1H, J = 4.2), 5.0 (m, 1H), 4.58−4.18 (9H). ^{31}P NMR: 0.51, −10.81. HRMS: 760.231 (M + 1).

5-Methyl-NAADP (21). The pH of a mixture of 5-methylnicotinic acid (**3b**) (70 mg, 0.51 mmol) and water (4 mL) was adjusted to 4.0 with 0.5 M NaOH, and the mixture formed a solution. Two mL of this solution was added to another vial, followed by solid NADP (16 mg, 0.021 mmol) and *Aplysia* cyclase enzyme (60 μL, 0.25 U). The pH of the solution was not adjusted again and was buffered by the 5-methylnicotinate solution. The solution was stirred for 2.5 h at 37 °C. The reaction was monitored by an assay to determine the amount of NADP left in the reaction. Upon completion, the catalyst was removed from the reaction solution by centrifuging in an Amicon Ultra centrifugal filter device (10000 molecular weight cut) at the speed of 3200*g* for 15 min. The filtrate contained the product, and the retentate was discarded. The clear filtrate was diluted, adjusted to pH 7.0, and purified by anion exchange chromatography on DE-52 cellulose. A gradient formed between 10−600 mM NH_4HCO_3 (700 mL) was applied and the product eluted into ca. 300 mM NH_4HCO_3. The appropriate fractions were lyophilized to obtain product as white solid (9.6 mg, 59%). ^{1}H NMR: 8.91 (s, 1H), 8.80 (s, 1H), 8.58 (s, 1H), 8.41 (s, 1H), 8.13 (s, 1H), 6.15 (d, 1H, J = 4.8), 5.93 (d, 1H, J = 4.2), 5.05 (m, 1H), 4.18−4.60 (9H), 2.48 (s, 3H). ^{31}P NMR: 0.67, −10.60. HRMS: 759.031 (M + 1).

5-Carboxy-NAADP (22). 3,5-Pyridine-dicarboxylic acid (**3d**, 70 mg, 0.42 mmol) was mixed in distilled water (6 mL). The mixture was stirred at 37 °C for 1 h and the pH was adjusted to 4.0 with 0.5 M KOH, and stirring continued at 37 °C for 0.5 h. The pH had decreased to around 3.5 as the solid of the mixture was slowly dissolving. The pH was again adjusted to 4.0, and this was repeated until the mixture had formed a clear solution. To this solution, solid NADP (16 mg, 0.02 mmol) was added, followed by *Aplysia* cyclase enzyme (100 μL, 0.42 U). The pH was not readjusted and was buffered by nicotinate solution. The reaction was stirred for 3.5 h at 37 °C. The reaction was monitored by an assay to determine the amount of NADP left in the reaction. Upon completion, the catalyst was removed from the reaction solution by centrifuging in an Amicon Ultra centrifugal filter device (10000 molecular weight cut) at the speed of 3200*g* for 15 min. The filtrate contained the product, and the retentate was discarded. The clear filtrate was diluted, adjusted to pH 7.0, and purified by anion exchange chromatography on DE-52 cellulose. A gradient of 10−600 mM NH_4HCO_3 (700 mL) was applied and the product eluted at around 500 mM NH_4HCO_3. The appropriate fractions were lyophilized to obtain product as white solid (5.5 mg, 32.4%). ^{1}H NMR: 9.41 (s, 2H), 9.24 (s, 1H), 8.54 (s, 1H), 8.38 (s, 1H), 6.22 (d, 1H, J = 5.2), 6.14 (d, 1H, J = 5.6), 5.1 (m, 1H), 4.62−4.18 (9H). ^{31}P NMR: 0.26, −10.1. HRMS 811.0353 (M + Na).

5-Ethyl-NAADP (23). The pH of a mixture of 5-ethylnicotinic acid (**3h**, 20 mg, 0.13 mmol) and water (2 mL) was adjusted to 4.0 with 0.5 M NaOH, and the mixture turned into solution. This solution was then added to another vial, followed by solid NADP (8 mg, 0.01 mmol) and *Aplysia* cyclase enzyme (60 μL, 0.25 U). The pH of the solution was not adjusted again and was buffered by the 5-methylnicotinate solution. The solution was stirred for 2.5 h at 37 °C. The reaction was monitored by an assay to determine the amount of NADP left in the reaction. Upon completion, the catalyst was removed from the reaction solution by centrifuging in an Amicon Ultra centrifugal filter device (10000 molecular weight cut) at a centrifugal force of 3200*g* for 15 min. The filtrate contained the product, and the retentate was discarded. The clear filtrate was diluted, adjusted

to pH 7.0, and purified by anion exchange chromatography on DE-52 cellulose. A gradient of 10−600 mM NH_4HCO_3 (700 mL) was applied and the product eluted at around 300 mM NH_4HCO_3. The appropriate fractions were lyophilized to obtain product as white solid (4.0 mg, 59%). ^{1}H NMR: 8.92 (s, 1H), 8.75 (s, 1H), 8.63 (s, 1H), 8.40 (s, 1H), 8.07 (s, 1H), 6.1 (d, 1H, J = 4.8), 5.93 (d, 1H, J = 4.2), 5.05 (m, 1H), 4.18−4.60 (9H), 2.78 (q, 2H, ^2J = 15.0, ^3J = 7.2), 1.20 (t, 3H, J = 7.2). ^{31}P NMR 0.86, −10.80. LC-MS (ESI) m/z 773.1 (M + 1).

5-Azido-NAADP (24). 5-Azidonicotinic acid HCl (**3i**·HCl, 35 mg, 0.42 mmol) was mixed in distilled water (3 mL). The mixture was stirred at 37 °C for 1 h, and the pH was adjusted to 4.0 with 0.5 M KOH and again stirred at 37 °C for 0.5 h. The pH had decreased to around 3.6 as the solid phase slowly dissolved. The pH was again adjusted to 4.0, and this was repeated until the mixture had formed a clear solution. To this solution, NADP (10 mg, 0.013 mmol) and *Aplysia* cyclase enzyme (80 μL, 0.33 U) were added. The reaction was buffered by nicotinate solution and was stirred for 3.5 h at 37 °C. The reaction was monitored by an assay to determine the amount of NADP left in the reaction. Upon completion, the catalyst was removed from the reaction solution by centrifuging in an Amicon Ultra centrifugal filter device (10000 molecular weight cut) at the speed of 3200g for 15 min. The filtrate contained the product, and the retentate was discarded. The clear filtrate was diluted, adjusted to pH 7.0, and purified by anion exchange chromatography on DE-52 cellulose. A gradient of 10−600 mM NH_4HCO_3 (700 mL) was applied and the product eluted at around 320 mM NH_4HCO_3. The appropriate fractions were lyophilized to obtain product as white solid (6.5 mg, 61%). ^{1}H NMR: 8.82 (s, 2H), 8.60 (s, 1H), 8.48 (s, 1H), 8.41 (s, 1H), 8.12 (s, 1H), 6.12 (d, 1H, J = 6.0), 5.92 (d, 1H, J = 5.4), 5.05 (m, 1H), 4.60−4.16 (9H). ^{31}P NMR: 0.41, −10.92. LC-MS (ESI) m/z 786.1 (M + 1).

Biological Testing. Testing of NAADP derivatives for Ca^{2+} release on cell free receptor systems were performed on homogenates (1.25% v/v) prepared from sea urchin eggs (*Strongylocentrotus purpuratus*) diluted with intracellular medium containing 250 mM potassium gluconate buffer (pH 7.2), 0.5 mM ATP, 4 mM creatinine phosphate, creatinine kinase, and 3 μM fluorescent indicator, Fluo-3. The dilutions and all experiments were conducted at 17 °C. Fluo-3 is a calcium chelating indicator, which is nonfluorescent in absence of Ca^{2+} ions, but after binding Ca^{2+}, it emits fluorescence. This fluorescence can be measured using a fluorescence plate reader (excitation 490 nm and emission 535 nm, suitable to avoid interference from reduced pyridine nucleotide).[3,50,51]

When the homogenates were treated with NAADP agonists, the Ca^{2+} released from the sensitive stores was chelated by the indicator, leading to increase in fluorescence. This increase in fluorescence intensity was measured and was proportional to the Ca^{2+} concentration.

Receptor Binding Studies of the 4- or 5-Substituted NAADP Analogues. The competitive binding studies were performed according to our previously published procedures.[3] The binding assays were done in triplicate in 96-well filter plates containing the cell free sea urchin egg homogenate and constant concentration of radioligand [^{32}P]NAADP (0.2 nM). Seven concentrations of each compound were tested to determine their IC_{50}. The competitor and [^{32}P]NAADP were incubated simultaneously with the sea urchin egg homogenate for 90 min at 4 °C. The homogenate was filtered and washed and the radioactivity retained on the filter was determined by liquid scintillation.

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Supporting Information Available: Detailed information on synthetic methods, analytical, spectroscopic, and Ca^{2+} releasing activity. This material is available free of charge via the Internet at http://pubs.acs.org.

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