Chapter 2

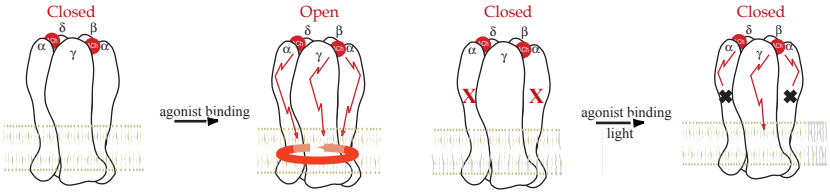
Synthesis and Uses of 5-(*o*-nitrobenzyl)selenyl-2-hydroxypentanoic acid (NBSeOH)

2.1 Introduction:

As described in the introduction, 5-(*o*-nitrobenzyl)selenyl-2-hydroxypentanoic acid (NBSeOH) is designed to cleave the backbone of a protein site-specifically in real time. This ability allows an experimenter to probe many structure-function relationships of a protein. Figure 2.1 gives a good depiction of three different aspects of ion channel function that could be explored using light-activated protein cleavage.

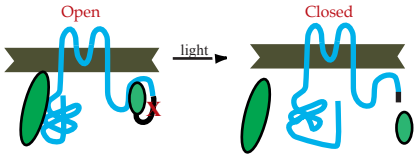
It is rather difficult to map the protein backbone conformational changes that occur during opening and closing of a channel. By incorporating a cleavable amino acid, the function of a channel can be studied before and after photolysis. The technique can be used to incorporate the cleavable amino acid and map the backbone positions where conformational changes occur during the opening and closing of the channel. For instance, the cleavable amino acid could be used to uncouple the binding of an agonist to the opening of the channel. In addition, the functional interactions of regulatory proteins could be studied. This could be done by creating a fusion protein of the ion channel to the regulatory protein with the cleavable amino acid incorporated into the tether and analyzing the function of the channel before and after photolysis. The technique could also be used to identify protein domains that are essential in function. A photo-cleavable amino acid allows one to proteolyze a protein site-specifically *in vivo*.

1) Distinguish where the conformational change occurs down the backbone of a protein.



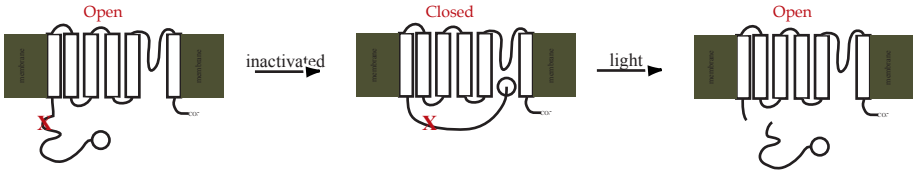
Representation of the nAChR with the backbone of the receptor thought to move during receptor opening (colored in red). If this backbone was broken using a light-activated cleavable amino acid, the channel would not function

2) Study regulatory proteins by covalently linking them to the protein of interest and then cleaving the tether.



Many ion channels are modulated by interactions with regulatory proteins. The green ellipses are regulatory proteins and the blue is an ion channel. The channel's function can be tested bound and unbound to the tethered regulatory protein.

3) Distinguish if parts of a protein are essential in function.



Schematic of the Shaker B ion channel. The N-terminus ball is depicted as a circle. The N-terminus is known to inactivate the channel by blocking the pore. If the N-terminus was clipped off the protein, inactivation would not occur.

X = cleavable amino acid

Figure 2.1 Uses of a photo-cleavable amino acid

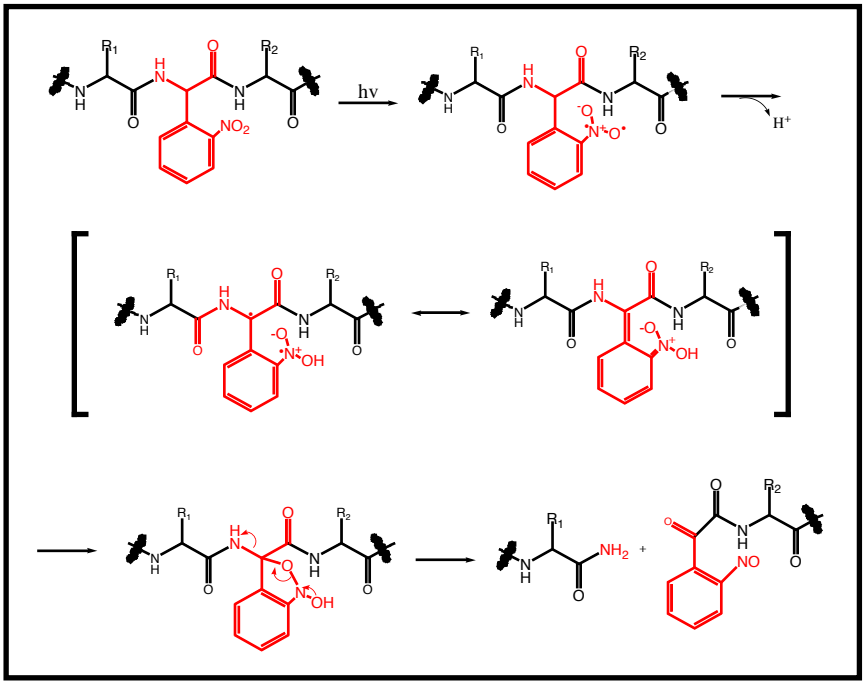


Figure 2.2 Nitrophenylglycine (Npg)

The first attempt to create a light-induced cleavable amino acid was the synthesis of (2-nitrophenyl)glycine (Npg) (Figure 2.2) by Dr. Pam England (1). Irradiation of Npg leads to site-specific, nitrobenzyl-induced photochemical proteolysis (SNIPP). The amino acid is based on nitrobenzyl photochemistry. Nitrobenzyl and analogs of it are used as protecting groups in organic synthesis, (2-5) and in biology they are used to produce 'caged' agonists, antagonists, and second messengers that are liberated photochemically (6-8). Dr. England incorporated the amino acid Npg into the Shaker B potassium channel and the nicotinic acetylcholine receptor *in vivo* and showed that irradiation *in vivo* of the proteins led to SNIPP.

The Shaker B channel is a homotetrameric voltage activated K^+ channel (Figure 2.1, Section C). In this channel the N-terminus is known to inactivate the channel after opening by blocking the pore of the channel on a millisecond time scale; this inactivation is called N-type inactivation (9-12). A "ball-and-chain" mechanism underlies N-type inactivation. The first 20 amino acids in the N-terminus form a structural domain or "ball" region that is connected to the rest of the channel by a "chain" sequence of 60 or more amino acids that tethers the inactivation ball to the rest of the protein. Deletion of a section of the N-terminus (Δ6-46) completely abolishes N-type inactivation. Dr. England showed that photolysis of the channel with Npg incorporated into the N-terminus at sites Leu 47 or Pro 64 in the channel decreases the N-type inactivation (1). Photolysis of the Npg containing channels leads to loss of the inactivation ball (N-terminus domain) of the channel, which leads to reduction of inactivation. It has been shown previously that only a single ball is necessary for channel inactivation, therefore, because there are four

independent balls in the channel the rate and extent of inactivation depends on the number of balls that the channel contains. Dr. England found that on average two of the four balls from ShB channels were removed after a 4-hour irradiation (1).

The Npg work illustrates well the power cleavable amino acids can have. However, there were several problems with Npg. Probably due to the steric bulk at the α carbon on Npg, the unnatural amino acid did not suppress well into ion channels (reduced incorporation)(13). In suppression experiments with Npg at the αV132TAG site in the nicotinic acetylcholine receptor (nAChR), oocytes had to be injected three times before electrophysiological recordings could be done. In addition, for full cleavage to occur oocytes had to be irradiated for four hours. The long irradiation time reduced the utility of Npg because oocytes could not be irradiated during an electrophysiology recording. The NBSeOH work was initiated to try to find a molecule with more favorable suppression properties and more rapid photolytic kinetics than Npg.

NBSeOH (Figure 2.3) is a hydroxy acid whose side chain contains a three carbon tether and selenium that is protected with *o*-nitrobenzyl. Upon irradiation, the *o*-nitrobenzyl protecting group is photochemically removed to reveal a selenium anion. The selenium anion then attacks the carbinol carbon and initiates an intramolecular S_N2 displacement that cleaves the backbone ester. Selenium anions are known to be strong nucleophiles (14). Selenium has been shown in many model experiments to attack the carbinol carbon and break sterically bulky esters (15-18). Selenocysteine is a naturally occurring amino acid. The pK_A of selenocysteine is 5.73 (19); therefore, after photolysis selenium should be in its anionic state. In addition, large hydrophobic unnatural amino

acids and hydroxy acids have been shown to suppress rather well with the nonsense suppression technique (13, 20-22). The □ carbon on NBSeOH is not hindered. Based on all of the above reasons, it is believed that NBSeOH will suppress better and break the peptide backbone quicker than Npg.

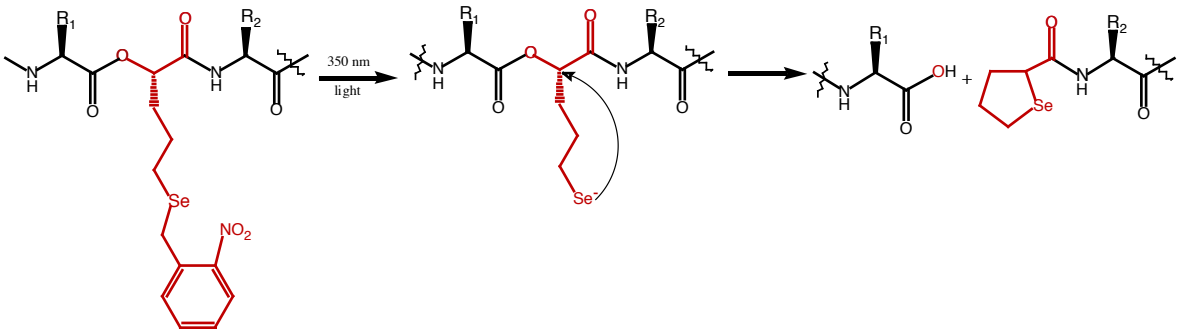


Figure 2.3 Photochemical cleavage of NBSeOH

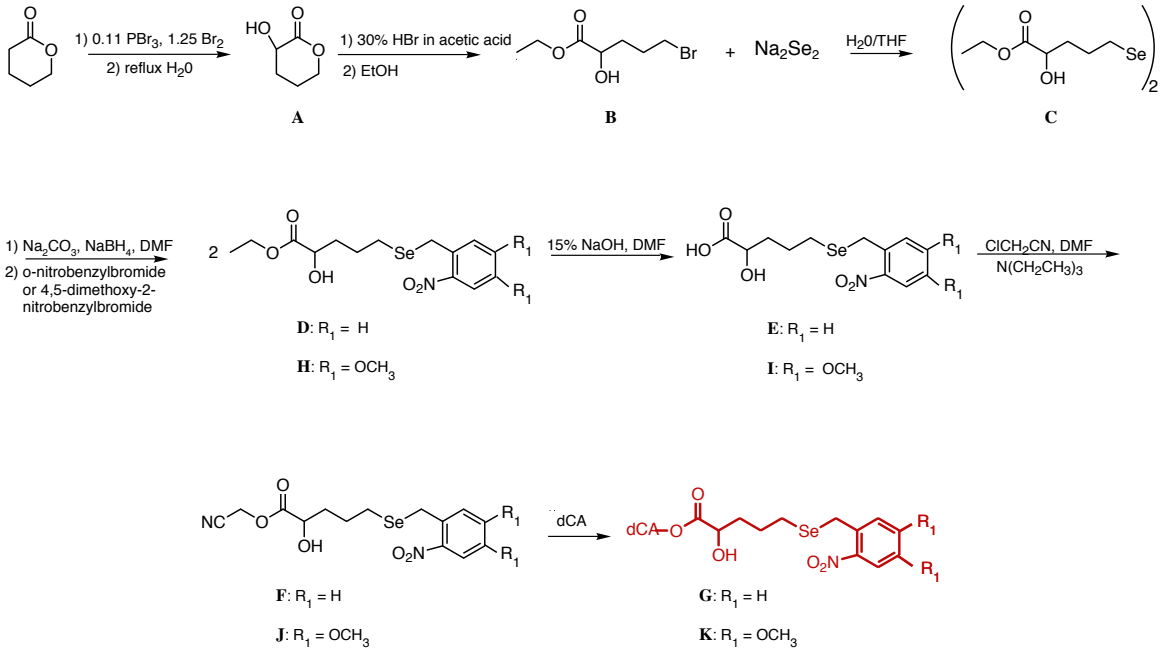


Figure 2.4 Synthetic route to dCA-NBSeOH and dCA-NVSeOH

2.2 Synthesis:

2.2.1 NBSeOH and NVSeOH:

The synthetic scheme which now seems straightforward took several iterations before the molecule was finally isolated (Figure 2.4). Some of the initial pathways that were used to obtain the molecule are discussed in the appendix. The final synthetic route is shown in Figure 2.4. The route begins by the conversion of δ-valerlactone to 2-hydroxy δ-valerlactone using the method described in (23). The lactone was then opened using 30% HBr in acetic acid (24, 25). The brominated product was then reacted with sodium diselenide to synthesize the 2-hydroxypropylethyl ester diselenide according to literature protocol (26). The diselenide was reduced with sodium borohydride, which revealed a selenium anion that was reacted with *o*-nitrobenzyl bromide (27, 28). The ethyl ester protecting group was removed and the carboxylic acid was activated using chloroacetonitirile and ligated to dCA as described in (29-31).

In addition, the nitroveratryl version of NBSeOH or 5-(4,5-dimethoxy-2-nitrobenzyl)selenyl-2-hydroxypentanoic acid (NVSeOH) was synthesized. The synthetic route described above was used except during the reduction of the diselenide 4,5-dimethoxy-2-nitrobenzyl bromide was used. NVSeOH was also ligated to dCA but has not been as extensively tested as NBSeOH.

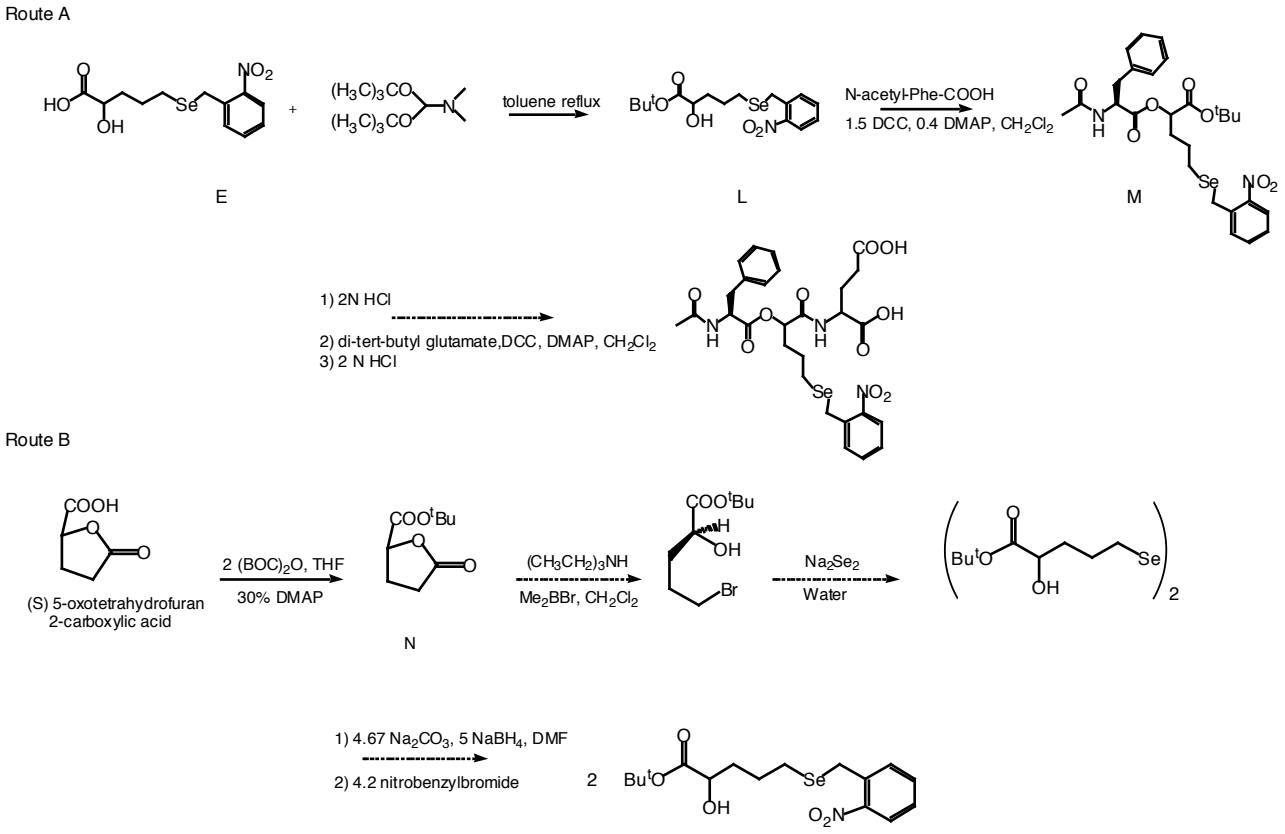
All products were characterized by NMR and mass spectrometry. Selenium compounds have a nice diagnostic pattern in mass spectrometry. The isotopic masses for selenium are 79.9 (100%), 77.9 (47.9%), 75.9 (18.9%), 81.9 (17.6%), and 76.9 (15.4%).

Therefore, it is very easy to visualize the isotopic pattern of selenium in a mass spectrum. This was used quite often to visualize if the crude material did contain product. The experimental section contains all NMR and mass spectra, and in many of the selenium containing compounds the isotopic pattern found in the mass spectrum is listed.

2.2.2 Peptide synthesis:

Initially, a synthetic scheme was derived to synthesize a tripeptide (Phe-NBSeOH-Glu) that could then be used in cleavage studies. We thought that a peptide would be easier to handle than a full protein to study if NBSeOH did indeed cleave an ester after photolysis. NMR and mass spectrometry analysis would reveal if NBSeOH was cleaving. The synthetic scheme for the tripeptide is shown below (Figure 2.5). The synthesis was never carried out any further than the phenylalanine coupling because the yield of the *tert*-butylation synthetic step was low.

N,N-dimethylformamide di-*tert*-butyl acetal was used to esterify the carboxylic acid of NBSeOH (Figure 2.5, Route A). This reagent allows *tert*-butylation to occur only on the carboxylic acid and not the alcohol (32). Ditertbutyldicarbonate (BOC_2O) with a catalytic amount of dimethylaminopyridine was used initially but was found to protect both the alcohol and the carboxylic ester (33). However, the N,N-dimethylformamide di-*tert*-butyl acetal reaction gave a significant amount of selenium eliminated product (2-hydroxy-pent-4-enoic acid *tert*-butyl ester), and this side reaction significantly reduced the yield of the final dipeptide.



Synthesis of peptide with either N,N'-dimethylformamide di-*tert*-butyl acetal (Route A) or dimethylboron bromide (Route B).

Figure 2.5

In the N,N-dimethylformamide di-*tert*-butyl acetal reaction a large excess (4 equivalents) of the reagent is used because the *tert*-butyl carbocation that forms in the reaction can either react with the carboxylic acid on NBSeOH or can lose a proton and form 2-methyl-2-propene (32). However, in the present system the selenium could also be alkylated by the *tert*-butyl cation, which then leads to the selenium eliminated product (2-hydroxy-pent-4-enoic acid *tert*-butyl ester) (34). The reaction was done in both refluxing toluene and at room temperature. The eliminated product was still a major product in the reaction when it was run at room temperature. The reaction has never been performed with just one or two equivalents of N,N-dimethylformamide di-*tert*-butyl acetal; using a smaller excess of the reagent might prevent the elimination of the selenium in the desired product but will probably reduce the yield of the reaction. The new synthetic route (B) in Figure 2.5 protects the carboxylic acid early in the synthesis and eliminates the N,N-dimethylformamide di-*tert*-butyl acetal reaction.

The protected NBSeOH was then coupled to N-acetyl protected phenylalanine using 1,3-dicyclohexylcarbodiimide and a catalytic amount of dimethylaminopyridine (35). Three milligrams of the dipeptide was synthesized. The dipeptide was used in a photolysis NMR experiment. The products of the photolysis were isolated using Prep-TLC and analyzed by a mass spectrometer. This experiment will be discussed in the results section.

A new synthetic route has been designed to synthesize *tert*-butyl NBSeOH (Figure 2.5, Route B). This synthetic route starts with (S)-oxotetrahydrofuran 2-carboxylic acid. The carboxylic acid is protected with ditertbutyldicarbonate (BOC_2O)

and the furan ring is opened with dimethylboron bromide (33, 36). The rest of the synthesis is similar to the synthetic scheme in Figure 2.4. I have synthesized 5-oxotetrahydrofuran 2-carboxylic acid *tert*-butyl ester in a prior synthetic route (See Appendix). The reaction goes in a 91% yield and several grams of the product have been synthesized. The rest of the synthesis will be carried out by another member of the Dougherty group. Hopefully with this new synthetic route, the tripeptide can be synthesized efficiently.

2.3 Results:

2.3.1 Dipeptide model system:

A dipeptide containing phenylalanine and NBSeOH was synthesized using routine solution phase peptide coupling reactions. Due to the low yield of the *tert*-butylation, the final amount of dipeptide synthesized was approximately 3 milligrams. The peptide was dissolved in 400 μl of deuterated acetonitrile and used in NMR studies. Argon was bubbled through the sample for several minutes before the sample was capped. The NMR tube was placed such that the beam of light from the arc lamp encompassed the whole four inches of the sample. The arc lamp was filtered to transmit 330-370 nm light with maximum intensity at 350 nm. The sample was irradiated for 30s and then for four one-minute time points, consecutively. After each time point, an NMR spectrum was taken. Reference (37) was used as a guide to create the photolysis protocol.

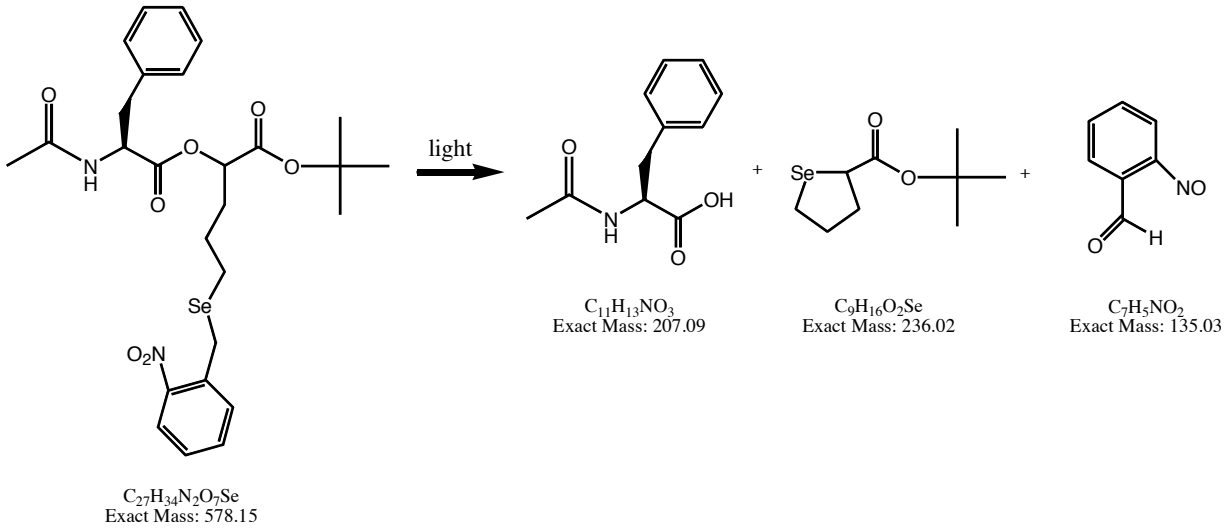


Figure 2.6

$C_9H_{16}O_2Se$ Theoretical isotopic pattern					
238 (17.6%)	237 (10%)	236 (100%)	235 (5.1%)	234 (47.9%)	233 (15.4%)
Experimental values					
238 (13%)	237 (42%)	236 (100%)	235 (65%)	234 (65%)	233 (41%)

Table 2.1

When NBSeOH is deprotected, the nitrobenzyl protecting group forms nitrosobenzylaldehyde (Figure 2.6). An aldehyde peak at 10.15 ppm begins to emerge in the spectra after 2.5 minutes. In addition, the benzyl peak at 4.1 ppm begins to change form and diminishes in size. However, an internal standard was not used in the sample, and therefore the exact difference in integration of peaks between different time points is hard to evaluate. After the last time point, Prep-TLC was used to separate the products of the photolysis. Three ultraviolet active bands were seen at Rfs of 0.56 (band 1), 0.77 (band 2), and baseline (band 3). Bands were cut out of the plate, and the products extracted out of the silica with ethyl acetate.

All three products were run on both the electrospray (ESI) mass spectrometer and gas chromatograph – mass spectrometer (GC-MS). Band 1 had a mass value of 206 $[M-H]^-$. This mass value corresponds to N-acetyl-phenylalanine, which would arise if cleavage of the ester occurred. Band 2 when injected onto the GC-MS produced a broad peak from 16.50-20 minutes. The major area of the peak between 17.5-19 minutes gave mass to charge ratios of 236, 252, and 264. The 236 mass corresponds to the selenium product that occurs when the ester in the peptide is broken (Figure 2.6). The 252 mass is (236 + 16) and 264 mass is (236 + 18), which could correspond to an added oxygen or water on the molecule. All three masses have n-2 and n+2 peaks, which correspond to a selenium isotopic pattern. However, the isotopic ratios found in the 236 mass do not completely match the theoretical isotopic pattern for the molecule (Table 2.1). While the origin of the large M+1 and M-1 peaks is unexplained at present, large M-2 and M+2

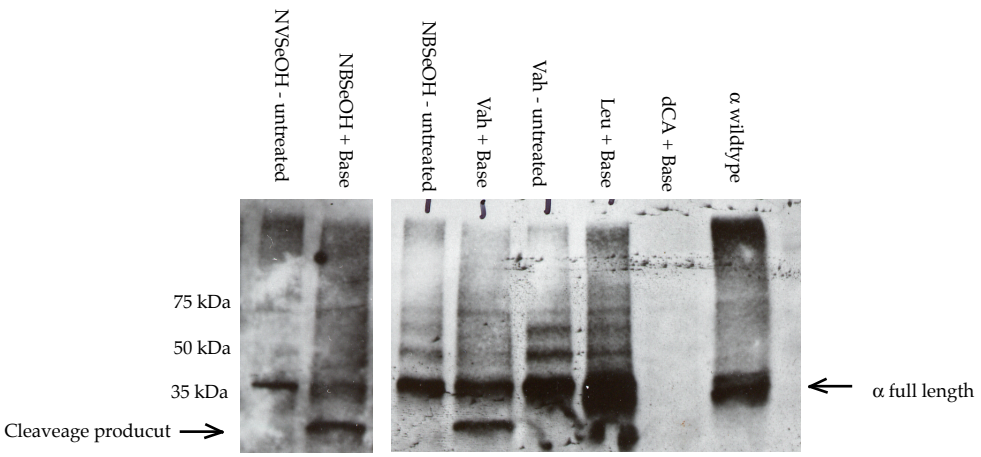
peaks are not seen in the GC-MS spectra of molecules not containing selenium. No mass spectral peaks were seen with the baseline band (3) using electrospray or the GC-MS. These preliminary results are very promising, and it definitely seems like photolysis and cleavage are occurring. However, the experiment should be done again with a larger quantity of material. In addition, an internal standard should be included in the sample (either pentachloroethane that has singlet at 6.39 ppm or tetrachloroethane that has a singlet at 6.08 ppm). With the new synthetic scheme using dimethylboron bromide, more material should be able to be synthesized.

2.3.2 *In vitro* protein incorporation – base hydrolysis:

A quick and easy technique to visualize if an unnatural amino acid is translationally competent and is incorporating into a protein is through *in vitro* transcription. Rabbit reticulocyte lysate was used to *in vitro* transcribe mRNA coding for nAChR □L250TAG subunit. This construct also contained an HA epitope between residues 347-348. The HA epitope is an antigenic peptide (YPYDVPDYA) derived from hemagglutinin (38, 39). This epitope was used to visualize the protein on a Western blot using antibodies against the HA sequence. Full length protein was found in lanes containing mRNA and NBSeOH charged tRNA. No protein was seen in lanes for which the transcription mix contained mRNA only or mRNA with uncharged tRNA (labeled dCA control) (blot 2.2).

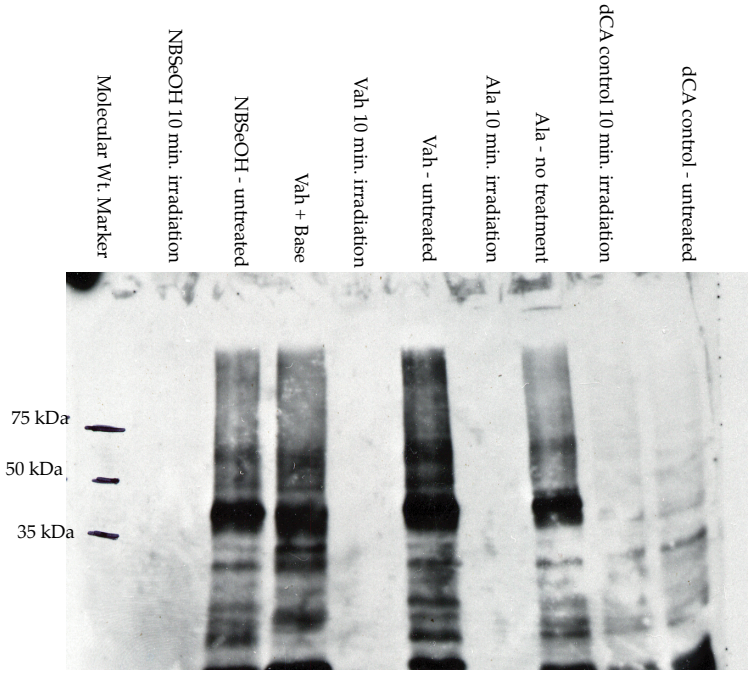
Hydroxy acids form ester bonds when incorporated into proteins. The ester linkage can be hydrolyzed using concentrated base and the cleaved product may be

observed on a Western blot (20). Blot 2.1 is a Western blot of nAChR δ subunit suppressed with Leu, β-hydroxy valine (Vah), NBSeOH, and NVSeOH. The arrow indicates the cleaved product band, which is observed only in the Vah and NBSeOH base treated lanes. In blot 2.1, the NVSeOH base treated lane is not shown because the sample was poorly loaded in that lane. However, NVSeOH has been shown to cleave under base hydrolysis conditions when incorporated into the δ 247 TAG mRNA construct (data not shown). Western blot 2.1 verifies that NBSeOH and NVSeOH are tolerated by the ribosome and are being incorporated into the protein. Based on relative intensities of the full protein on the Western blots, suppression efficiency is Vah > Leu > NBSeOH > NVSeOH.



In vitro suppression in the α subunit at position 250, protein contains the HA epitope. Samples were treated with base or loaded as is (untreated). See methods section for exact protocol.

Blot 2.1



In vitro suppression in α subunit, containing the HA epitope and seven histidines tag, at position 247. Protein was purified using Ni^{2+} beads and eluded using 300 mM imidazole. The samples were irradiated for ten minutes or loaded as is (untreated).

Blot 2.2

2.3.3 *In vitro* protein incorporation – photolysis experiments:

The ability of NBSeOH to photochemically cleave the protein backbone was also analyzed using *in vitro* transcribed protein. NBSeOH was suppressed in the δ subunit of the nAChR at position 247 using rabbit reticulocyte lysate. This construct also contained the HA epitope. Western blot analysis was used to view the protein. If cleavage of the ester does occur after photolysis, a band corresponding to 24 kDa will be seen. This protein fragment will contain the amino acids from 247 on to the end of the protein (including the HA epitope). In addition, if transcription stopped at the UAG codon at position 247 the protein fragment would not be seen on the Western blot because the HA epitope is between positions 347-348.

Photolysis experiments were first tried on protein in the rabbit reticulocyte lysate mix. Sample treatment can be found in the methods section. Samples were irradiated for ten minutes using our standard procedure (31, 40). No cleavage bands were seen on the gel. Rabbit reticulocyte lysate is a dark red color due to the high quantity of heme in the lysate. There was concern that the lysate was absorbing much of the light, and effectively abrogating photolysis of NBSeOH.

Based on the photolysis experiments, His tag purification was performed to remove the δ subunit from the lysate mix after transcription. Seven histidines were cloned into the C-terminus of the δ HA 247 TAG construct. The polyhistidine tag is used to isolate protein using Ni^{2+} agarose beads (41-43). Protein is removed from the beads using a high concentration of imidazole. In my procedure, a 300 mM imidazole concentration was used to elude protein off the beads. Photolysis experiments were

performed with protein in the 300 mM imidazole solution (Blot 2.3). As can be seen in the Western blot, protein seems to disappear after being exposed to light (no matter what amino acid was suppressed). I believe the high concentrations of imidazole in the samples resulted in aggregation and/or degradation of the protein during the irradiation. Consequently, imidazole must be removed before the photolysis experiments can be performed.

There are many ways to remove small molecules from a protein sample. Often dialysis is used to remove salts. However, because my samples are very small (150 l), dialysis is a rather tricky process. Instead of dialysis, a spin column containing a semi-permeable membrane (molecules $\geq$ 30 kDa retained) from Millipore was used. The imidazole samples were concentrated and the imidazole removed using the spin-filter columns. The purified protein was then used in photolysis experiments. Several photolysis experiments were run, but the photolytic fragment was seen in only one experiment (data not shown).

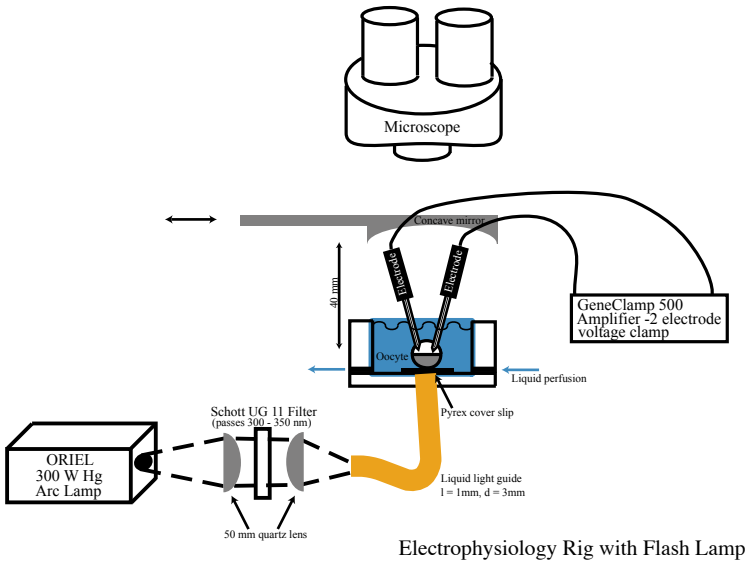
The photolysis *in vitro* experiments have not been as convincing as the base hydrolysis experiments. Often a non-specific band is seen around 24 kDa on Western blots of the transcribed protein; and therefore, it is difficult to distinguish if the band is due to photolysis or is the non-specific band. The non-specific band is seen in dCA control and mRNA only translation reactions; therefore, it is not thought to be due specifically with the protein. However, the band does get darker when a protein is expressed in the reaction. Another position closer to the N-terminus of the protein than 247 could be used for suppression experiments, allowing a larger cleavage product to be

produced upon photolysis. For instance, a band around 35 kDa would be easier to recognize (no non-specific bands). However, a nice method for purification using Ni^{2+} beads and a spin-filter has been developed, and hopefully, using this method, a Western blot of photo-cleaved protein can be obtained.

2.3.4 *In vivo* protein incorporation:

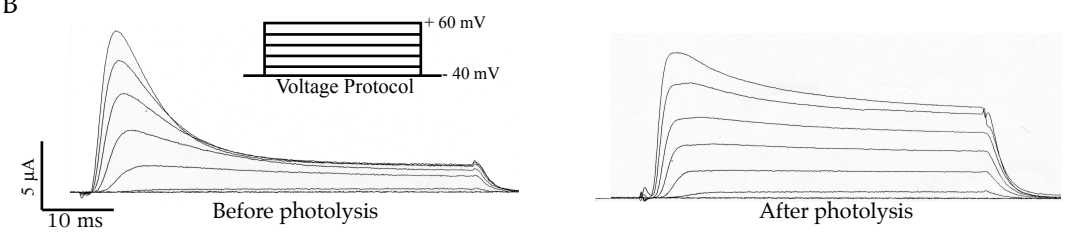
NBSeOH has been incorporated into the Shaker B potassium channel. When the channel opens due to depolarization, potassium ions flow out of the cell through the channel. By convention, this current is illustrated has a positive current. One of the characteristics of this voltage-gated channel is rapid (N-type) inactivation which results from the N-terminal portion of the receptor blocking the open pore (9-12). This inactivation can be seen in Figure 2.7 (before photolysis trace). The current decreases in milliseconds after opening of the channel. Previous experiments have shown that removal of the N-terminus of the receptor produces a channel that does not inactivate. Using *in vivo* nonsense suppression, NBSeOH, Leu, and Npg were introduced into the N-terminus at position 47 in the Shaker B potassium channel. This is the same position that Pam England used in her Npg study (1). Suppression efficiency with NBSeOH was quite good, and importantly, was roughly 10-fold greater than with Npg.

A



Electrophysiology Rig with Flash Lamp

B



Electrophysiology traces of ShBL47TAG mutants with NBSeOH incorporated. Oocytes are irradiated with light for one minute on electrophysiology rig, before the second trace is taken (after photolysis trace).

C

$$\% \text{ of Noninactivating current} = \frac{(\text{Amplitude of current} \approx 35 \text{ ms after Voltage Jump})}{(\text{Maximum Amplitude of Current})} \times 100$$

$$\% \text{ of Inactivating current} = 100 - \% \text{ of Noninactivating Current}$$

Figure 2.7

Experiments were done on a two-electrode voltage-clamp oocyte rig equipped with a flash lamp that is connected to the bath of the rig with a liquid light guide (Figure 2.7A) (44). When the shutter is open the oocyte is bathed in light. As shown in Figure 2.7B, one minute of photolysis leads to a high percentage of noninactivating current (or reduction of inactivating current). Preliminary analysis suggests that on average the percent of noninactivating current is 35 $\pm$ 3% in Shaker B channels suppressing NBSeOH after a one minute photolysis. However, some oocytes suppressing NBSeOH show higher percent of noninactivating current. Figure 2.7B is a trace of an oocyte suppressed with NBSeOH that has a 50% increase in the noninactivating current. Figure 2.7C illustrates the calculations that are used in analyzing the data. In the England paper, 27% noninactivating current is seen after a 4-hr photolysis in Shaker channels suppressing Npg (1).

2.4 Discussion and conclusions:

As revealed by the *in vitro* work, NBSeOH can be incorporated into the □ subunit of the nAChR at positions 250 and 247 using nonsense suppression. Efficiency of incorporation is lower than leucine and hydroxy-valine. This is probably due to the steric bulk of the hydroxy acid. However, *in vitro* incorporation of NBSeOH is better than Npg (data not shown). Base hydrolysis experiments unambiguously confirm the suppression with NBSeOH.

The preliminary dipeptide results reveal that the *o*-nitrobenzyl protecting group begins to be removed in 2.5 minutes (when the aldehyde peak emerges). In addition,

after 4.5 minutes, two products can be isolated from the photolysis reaction. The products have mass values corresponding to N-acetyl-Phe and tetrahydroselenophen-2-carboxylic acid *tert*-butyl ester, both of which would be created if the ester bond were cleaved. With the newly designed synthetic route B, the tripeptide study should be possible. The tripeptide study will hopefully confirm the preliminary dipeptide results.

The *in vivo* ShB experiments have also shown NBSeOH to be incorporated into a protein and to cleave a protein more efficiently than Npg. NBSeOH has been shown to cleave an ester when incorporated into the ShB channel in a one-minute irradiation. Comparable cleavage with Npg requires four-hour irradiation. However, it should be noted that Pam England used a different photolysis setup than the one used in these experiments (1). While more work remains to be done, these early studies indicate that 1) NBSeOH can be synthesized and efficiently incorporated into proteins expressed *in vitro* and *in vivo* 2) photolysis of peptides or proteins containing NBSeOH does lead to backbone cleavage 3) in terms of suppression and photochemical efficiency, NBSeOH represents a significant improvement over Npg.

2.5 Methods:

2.5.1 Synthesis:

Reagents were purchased from Aldrich, Sigma, or other commercial sources. All bought chemicals were used without purification except □-valerlactone, which was purified by distillation under vacuum at (bp 58-60ºC/0.5 mm Hg). Anhydrous solvents

were purchased from Mallinkrot Baker; anhydrous DMF was obtained from Fluka. Flash chromatography was done on 230-400 mesh silica gel with the solvent indicated. All NMR shifts are reported as δ ppm downfield from TMS. ^{1}H NMR and ^{13}C spectra were recorded using a GE QE-300 MHz spectrometer. Electrospray (ESI) ionization, quadrupole mass spectrometry was performed at the Caltech Protein/Peptide Micro Analytical Laboratory. Gas chromatography – mass spectrometry (GC-MS) was performed at Caltech mass spectrometry laboratory. High performance liquid chromatography (HPLC) separations were performed on a Waters dual 510 pump liquid chromatography system equipped with a Waters 490E variable wavelength UV detector. Semi-preparative samples were separated using a Whatman Magnum 9 column (9.4 x 500 mm, Partisil 10, ODS-3).

Synthesis of NBSeOH

2-hydroxy γ-valerlactone (A) – To a 25 ml round bottom with a condensor was added 3 ml of purified γ-valerlactone (32.3 mmol) and 0.34 ml of phosphorous tribromide (3.56 mmol) via syringe. The mixture was heated in an oil bath to 100ºC. The reaction was kept at this temperature for 10 minutes before 2.1 ml of bromine (40.4 mmol) was slowly added to the reaction via syringe. The reaction was kept under argon throughout the reaction. After complete addition of bromine, the reaction was heated to 120ºC for an hour and a half. The oil bath was then turned off and the reaction was opened to air to allow the removal of any excess bromine gas before quenching the reaction. The

reaction was then transferred to a 250 ml round bottom and 53.8 ml of water was added. The solution was then refluxed for two days. The solvent was then removed under vacuum. The dark brown oil, which remained after the solvent was removed, was then transferred to a 25 ml round bottom and product was purified using a Kugelrohr (99-101ºC, 0.8 mm Hg). After 3 hours, pure product was removed from the distillation bulbs. Yield: 62%. ^{1}H NMR (CD_2Cl_2) 10.9 (broad s, 1H), 4.45 (AB, *J* = 5.7, 3 Hz, 1H), 3.87 (m, 2H), 2.24 (m, 1H), 2.03 (m, 1H), 1.91 (m, 2H) ^{13}C (CD_2Cl_2) 177.3, 77.0, 70.2, 30.7, 25.9

2-hydroxy-4-bromopropylethyl ester (B) - In a 25 ml round bottom charged with product A (650 mg, 5.60 mmol), 1.67 ml of 30% HBr in acetic acid was slowly added via syringe. The reaction was heated to 70ºC for 12 hours. Then 6 ml of dry EtOH was added to the reaction. The reaction was heated another 12 hours at 70ºC. The reaction was quenched by adding several cubes of ice and 6 ml of saturated sodium bicarbonate solution. The product was extracted out of the aqueous solution using 3 X 15 ml of ether. The organic layers were combined, dried over $MgSO_4$, and decanted into a round bottom flask. The solvent was removed by vacuum and a 15 mm flash column was run in 60:40 ethyl acetate to hexane solvent system. Product is visualized on thin layer chromatography plates using $KMnO_4$ staining. Yield: 43%. ^{1}H NMR (CD_2Cl_2) 1.30 (t, J = 7.2, 3H), 1.80 (m, 4H), 3.42 (m, 2H), 4.12 (m, 1H), 4.24 (q, J=7.2, 2H) ^{13}C NMR (CD_2Cl_2) 174.9, 70.0, 62.1, 33.9, 33.1, 28.7, 14.3

2-hydroxypropylethyl ester diselenide (C) – In a 25 ml round bottom, elemental selenium (137 mg 1.73 mmol), $NaBH_4$ (131 mg, 3.45 mmol), and 10 ml of water were added. Elemental selenium was measured in a ventilated chemical hood. Elemental selenium is highly toxic and is easily taken up by the lungs. After 10 minutes, another 137 mg of elemental selenium was added to the reaction. The reaction was then slightly heated over a steam bath. After 7 minutes, the solution turned a dark brown which is indicative of Na_2Se_2 formation. Product B (773 mg) was dissolved in 4 ml of a 1:1 mixture of THF and water; the solution was added to the reaction. The reaction was stirred at room temperature overnight. The reaction was worked up with 4 ml of 1 N acetic acid and 10 ml of water. Product was extracted out of the aqueous solution with 4 X 10 ml CH_2Cl_2. The organic layers were combined, dried over $MgSO_4$, and decanted into a round bottom flask. The solvent was removed by rotary-evaporation. The product was used in this crude form in the further reactions. Product was rather unstable. After two weeks of storage, red elemental selenium began forming in the product. However, diselenide was purified for analytical analysis by flash chromatography using a 40:60 ethyl acetate and hexane solvent system. Yield: 91% ^1H (CD_2Cl_2) □ 4.19 (q, J = 7.2, 4H), 4.16 (m, 2H), 2.93 (m, 4H), 1.86 (m, 8H), 1.26 (t, J = 7.2, 6H). ^{13}C (CD_2Cl_2) □ 175.57, 70.53, 62.28, 34.56, 29.91, 26.91, 14.57. FAB-MS: for $C_{14}H_{26}O_6Se_2$ [M^-] calculated 451.0138, found [M^-] 451.0152.

ethyl 5-(*o*-nitrobenzyl)selenyl-2-hydroxypentanoate (D) – Diselenide product C (48.6 mg, 0.11 mmol), Na_2CO_3 (54.4 mg, 0.51 mmol), $NaBH_4$ (20.8 mg, 0.55 mmol), and 1.5

ml of anhydrous DMF were added to a 5 ml round bottom. The reaction was stirred at room temperature for 1 hour. The solution became light yellow over time. Nitrobenzylbromide (99.8 mg, 0.46 mmol) dissolved in 0.5 ml of anhydrous DMF was added to the reaction. The reaction was kept in the dark and stirred at room temperature overnight. The reaction was quenched with 2 ml of 1 N acetic acid and 7 ml of water. The product was extracted out of the aqueous solution using 2 X 10 ml ether. Solvent was removed by rotary-evaporation and high vacuum. A flash column was run using 40:60 ethyl acetate and hexane solution. Yield: 49%. ^{1}H (CD_2Cl_2) 7.96 (m, 1H), 7.52 (m, 1H), 7.37 (m, 2H), 4.18 (q, J = 7.2 Hz, 2H), 4.06 (m, 1H), 4.08 (s, 2H) 2.58 (m, 2H), 1.60 (m, 4H), 1.25 (t, J = 7.2 Hz, 3H) FAB-MS: for $C_{14}H_{19}NO_5Se$ [M^-] calculated 362.0507, found 362.0498

5-(*o*-nitrobenzyl)selenyl-2-hydroxypentanoic acid (E) – One milliliter of 15% NaOH solution and 0.5 ml of DMF were added to product D (39.2 mg, 0.11 mmol). The reaction was stirred for 6 hours. The reaction was quenched with 8 ml of 6 N HCl, and the product was extracted using 3 X 10 ml of ether. The organic layers were combined, dried over $MgSO_4$, decanted, and solvent was removed using rotary evaporation. Used without further purification. Crude yield: 92%. EIS-MS: for $C_{12}H_{15}NO_5Se$ [M^-] calculated 333.0 (100%), 359.0 (48%), 335.0 (17.7%); found [M-H]$^-$ 332.2 (100%), 330.0 (74%), 334.0 (18%)

5-(*o*-nitrobenzyl)selenyl-2-hydroxypentanoic acid cyanomethyl ester (F) – In the same round bottom containing selenide E (33.2 mg, 0.1 mmol), 1 ml of dry $ClCH_2CN$, 1 ml anhydrous DMF, and 37 μl (0.26 mmol) of dry triethylamine were added. The reaction was stirred for 36 hours. Then 15 ml of ether was added to the reaction, and the solution was washed with 7 ml of water. The organic layer was separated from the aqueous and the solvent removed by rotary-evaporation and high vacuum. Crude yield 69%. ^1H (CD_3CN) δ 7.85 (m, 1H), 7.60 (m, 1H), 7.45 (m,2H), 4.80 (s, 2H), 4.41 (s, 1.3H, $ClCH_2CN$), 4.08 (s, 2H), 4.07 (m, 1H), 3.44 (q, ether), 2.56 (m, 2H), 1.70 (m, 4H), 1.30 (m, ether) EIS-MS: for $C_{14}H_{16}N_2O_5Se$ $[M-Cl]^-$ calculated is 407.0 (100%), 405.0 (46.9%), 409.0 (43.2%); found $[M-Cl]^-$ 407.2 (100%), 405.2 (46.3%), 409.2 (45.1%)

5-(*o*-nitrobenzyl)selenyl-2-hydroxypentanoyl-dCA (G) – The cyanomethyl ester product F (12.75 mg, 35.8 μM) was coupled to the dinucleotide according to standard protocol (29, 30). In an oven-dried 5 ml conical flask with stir bar, cyanomethyl ester product F, *tetra*-n-butyl-ammonium salt of the dCA dinucleotide (14.4 mg, 11.9 μmol), and 0.25 ml of anhydrous DMF were added. After several hours, a catalytic amount of dry tetrabutylammonium acetate was added to the reaction. The reaction was stirred overnight. The crude product was purified by reverse-phase semi-preparative HPLC with a gradient from 25 mM NH_4OAc (pH 4.5) to CH_3CN. The desired fractions containing the hydroxy acid dinucleotide were combined, frozen, and lyophilized. The lyophilized solid was redissolved in 10 mM aqueous acetic acid/acetonitrile and lyophilized a second time to remove salts. The products were quantified by UV/Vis spectra and characterized

by mass spectrometry. EIS-MS: for $C_{31}H_{38}N_9O_{17}P_2Se$ [M^-] calculated is 950.09, found [M-H]$^-$ 950.2

Synthesis of peptide containing NBSeOH

t-butyl-5-(*o*-nitrobenzyl)selenyl-2-hydroxypentanoate (L) – To an oven dried 10 ml flask containing selenide E (70 mg, 0.21 mmol), 1 ml of toluene was added, and the reaction mixture was heated to 110ºC in an oil bath. When the oil bath reached 110ºC, 0.24 ml of N,N-dimethylformamide di-*tert*-butylacetal (0.84 mmol) was added to the reaction via syringe. The reaction was allowed to reflux for one and half hours. The reaction was then quenched with 14 ml of water and product was extracted out with one wash of 30 ml of ether and another wash of 10 ml of ether. The organic layers were combined and washed 2 X 10 ml of sodium bicarbonate. The organic layer was then dried over $MgSO_4$, decanted, and the solvent was removed by rotary-evaporation. A 15 mm column using 40:60 ethyl acetate to hexane solvent system was used. ^1H (CD_2Cl_2) □ 7.96 (m, 1H), 7.55 (m, 1H), 7.4 (m,2H), 4.23 (q, ethyl acetate), 4.09 (s, 2H), 4.07 (m, 1H), 2.57 (m, 2H), 2.11 (s, water), 2.00 (s, ethyl acetate), 1.53 (s, 9H), 1.20 (m, ethyl acetate). EIS-MS: for $C_{16}H_{23}NO_5Se$ [M^-] calculated is 389.1, found [M-Na]$^+$ 412.0.

Aceytl-NH-Phe-NBSeO-*tert*-butyl ester (M) – In a 5 ml conical flask containing □ 52 mg of product L (0.13 mmol), Aceytl-NH-PheCOOH (27.8 mg, 0.13 mmol), DCC (41.5 mg, 0.20 mmol), and DMAP (6.5 mg, 0.05 mmol) were added. One milliliter of dry methylene chloride was added to the reaction vessel. The reaction was stirred in the dark

for two days. The reaction was then brought up in 25 ml of ethyl acetate. The reaction mixture was gravity filtered. The effluent was then washed with 5 ml of sodium bicarbonate and 5 ml of salt solution. The solvent was then removed using rotary-evaporation and high vaccum. A flash column was run on crude material using a 60:40 ethyl acetate to hexane solvent system. ^1H (CD_2Cl_2) □ 7.99 (m, 1 H), 7.55 (m, 1H), 7.39 (m, 2H), 7.25 (m, 5H), 4.92 (m, 2H), 4.10 (s, 2H), 3.10 (m, 1H), 3.48 (m, 1H), 2.58 (m, 2H), 1.99 (s, 1.5H), 1.95 (s,1.5H), 1.65 (m, 4H), 1.49 (s, 4.5 H), 1.45 (s, 4.5 H), 1.1-1.4 (m – residual hexanes) FAB-MS: for $C_{27}H_{35}N_3O_7Se$ [M^+] calculated is 579.1609, found 579.1596.

Synthesis of dCA-NVSeOH

ethyl 5-(4,5-dimethoxy-2-nitrobenzyl)selenyl-2-hydroxypentanoate (H) –To 193 mg (0.43 mmol) of diselenide C, 213 mg (2.01 mmol) of sodium bicarbonate, 81.7 mg (2.16 mmol) $NaBH_4$, and 5 ml of anhydrous DMF were added. The reaction was allowed to stir for 1.5 hours. The solution turned from a dark yellow to a light yellow during the 1.5 hours. Then 500 mg (1.8 mmol) of 4,5-dimethoxy-2-nitrobenzylbromide was dissolved in 2 ml of anhydrous DMF and added to the reaction vessel. The reaction was stirred at room temperature for two days. The reaction was quenched with 8 ml of 1 N acetic acid and 20 ml of water. The product was extracted out of the aqueous solution using 4 X 40 ml of ether. The organic layers were combined and dried over Na_2SO_4 and decanted into a dry round bottom. The solvent was then removed by rotary evaporation and high

vacuum. Flash chromatography was performed using 50:50 ethyl acetate to hexane solvent system. Yield: 64%. ^{1}H NMR (CD_2Cl_2) □ 7.64 (s, 1H), 6.78, (s, 1H), 4.24 (m, 2H), 4.12 (s, 2H), 4.08 (m, 1H) , 3.94 (s, 3H), 3.90 (s, 3H), 2.60 (m, 2H), 1.80 (m, 4H), 1.30 (m, 3H). ^{13}C NMR (CD_2Cl_2) □ 113.56, 109.14, 70.36, 62.23, 56.87, 56.77, 34.96, 26.45, 25.26, 24.70, 14.59.

5-(4,5-dimethoxy-2-nitrobenzyl)selenyl-2-hydroxypentanoic acid (I) – Product H (216 mg, 0.55 mmol) was dissolved in 5 ml of 15% NaOH aqueous solution and 2.5 ml of DMF. The solution was stirred for six hours at room temperature. Reaction was kept in the dark the whole time. The reaction was quenched with 6 N HCl until pH reached □ 3.6. The product was extracted out of the aqueous solution with 3 X 30 ml of ether. The organic layers were combined, dried over $MgSO_4$. Solvent was removed by rotary evaporation. Crude Yeild: 70%. EIS-MS: for $C_{14}H_{18}NO_7Se$ [M^-] calculated is 393.03 (100%), 394.03 (48.0%); found $[M-H]^-$ 392.0 (100%), 393.2 (45.3%)

5-(4,5-dimethoxy-2-nitrobenzyl)selenyl-2-hydroxypentanyl cyanomethyl ester (J) – To an oven dried 5 ml conical flask with 66.2 mg (0.17 mmol) product I, 1 ml of chloroacetonitrile, 1 ml of anhydrous DMF, and 73.3 □l of triethylamine were added. The reaction was stirred for 36 hours. The reaction was brought up in 20 ml of ether and 10 ml of water. Product was extracted out with another 15 ml of ether. Ether layers were combined and concentrated by rotary-evaporation and high vacuum. Flash chromatography was done using ethyl acetate as the solvent system Yield: 60.8 mg,

83%. ^{1}H NMR (CD_2Cl_2) □ 7.62 (s, 1H), 6.77, (s, 1H), 4.82 (s, 2H), 4.11 (s, 2H), 4.09 (m, 1H), 3.92 (s, 3H), 3.89 (s, 3H), 3.88 (m, 2H), 2.90 (m,. 1H), 2.60 (m, 1 H), 1.80 (m, 4H). ^{13}C NMR (CD_2Cl_2) □ 113.6, 109.1, 70.4, 56.9, 56.8, 49.7, 43.6, 26.2, 25.3, 24.5. EIS-MS: for $C_{16}H_{20}N_2O_7Se$ [M^-] calculated is 432.0, found $[M-H]^+$ 433.0 and $[M-Na]^+$ 455.0

5-(4,5-dimethoxy-2-nitrobenzyl)selenyl-2-hydroxypentanoyl-dCA (K) – A 10 ml round bottom was charged with 14.1 mg (0.012 mmol) of dCA and 15 mg (0.35 mmol) of product J. Anhydrous DMF (500 □l) was added to the reaction vessel. The reaction was stirred for an hour and a half. No product was seen by analytical HPLC. Therefore, a catalytic amount of tetrabutylammonium acetate was added to the reaction. The reaction was allowed to stir overnight. The crude product was purified by reverse-phase semi-preparative HPLC with a gradient from 25 mM NH_4OAc (pH 4.5) to CH_3CN. The desired fractions containing the hydroxy acid dinucleotide were combined, frozen, and lyophilized. The lyophilized solid was redissolved in 10 mM aqueous acetic acid/acetonitrile and lyophilized a second time to remove salts. The products were quantified by UV/Vis spectra and characterized by mass spectrometry. EIS-MS: [M^-] calc'd for $C_{33}H_{43}N_9O_{19}P_2Se$: 1011.13 (100%), 1009.13 (48.4%); found $[M-H]^-$ 1010.2 (100%), 1008.2 (51%)

2.5.2 Dipeptide study:

Approximately 3 milligrams of Aceytl-NH-Phe-NBSeO-*tert*-butyl (M) described above were dissolved in 400 μl of deudrated acetonitrile. The solution was transferred to an NMR tube. Argon was bubbled through the sample for several minutes. The sample was capped and the top was wrapped with parafilm to seal the tube. All ^{1}H NMR spectra were taken on the Varian 500 MHz spectrometer. An initial ^{1}H NMR was taken before photolysis. Then, the NMR tube was placed such that the beam of light from the arc lamp (1000 W xenon arc lamp (Oriel) operating at 400 W equipped with WG-335 and UG-11 filters) encompassed the whole four inches of the sample. The sample was irradiated for 30s and then for four one minute time points, consecutively. After each time point, an NMR spectrum was taken. The reduction of the benzyl peak (4.1 ppm) and the appearance of an aldehyde peak (10.15 ppm) were the two specific peaks that were analyzed during the photolysis experiments.

After four and a half minutes of irradiation, the sample was purified by Prep-TLC. The TLC was developed using a 50:50 ethyl acetate and hexane solvent system. Three ultravoilet active bands were seen at Rfs of 0.77, 0.56, and baseline. Bands were removed using a razor blade and the product was extracted out of the silica using ethyl acetate. Samples were then analyzed by ESI ionization, quadrupole mass spectrometry and gas chromatography – mass spectrometry (GC-MS). The Hewlett Packard GC-MS was equipped with a HP-5HS column (30 m X 0.25 mm) and a 5970 mass selective detector. Reference (37) was used as a guide to establish a protocol for performing and analyzing peptide photolysis experiments.

2.5.3 *In vitro* protein incorporation – base hydrolysis:

In vitro transcription was carried out using Promega rabbit reticulocyte lysate translation system according to manufacturer's protocol and reference (20). The following reagents were combined and incubated at 30ºC for 2 hours – 8.75 µl of lysate mix, 0.25 µl Amino acid mix, 0.25 µl RNAse inhibitor, 1.75 µl water, 0.5 µl of mRNA (1 µg/µl for suppression experiments and 0.3 µg/µl for wildtype RNA), and either 1 µl of water or 1 µl of tRNA (1 µg/µl). Protein was then kept at –80ºC until further use.

Samples were made by taking 5 µl of *in vitro* translation mix and adding 5 µl of 4% SDS. This mixture was subdivided into two samples (untreated and base treated samples). Untreated samples were further dissolved in 1 µl of 10% SDS and then incubated for 1.5 hours at room temperature. After incubation, 1 µl of 10% SDS, 7 µl of water, and 14 µl of 2 X SDS loading buffer (100 mM Tris-Cl pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol) were added. Base treated samples were diluted with 2 µl of concentrated NH_4OH with 10% SDS, and the samples were incubated for 1.5 hours at room temperature. The reaction was then quenched with 1 µl of 30% acetic acid in 10% SDS. The samples were further diluted with 7 µl of water and 14 µl of 2 X SDS loading buffer. Samples (5 µl) were loaded onto 10-12 % Tris-Cl gels. After SDS-PAGE, the gels were transferred to nitrocellulose. The nitrocellulose paper was then blotted using a mouse anti-HA antibody and subsequently treated with a goat anti-mouse secondary antibody conjugated to horseradish-peroxidase for detection by chemiluminescence.

2.5.4 *In vitro* protein incorporation – photolysis:

Protein was synthesized according to the procedure described above (2.5.3) . 10 – 15 μl of sample was irradiated individually using the arc lamp (1000 W xenon arc lamp (Oriel) operating at 400 W equipped with WG-335 and UG-11 filters). For irradiations over ten minutes, eppendorfs were kept in water. In experiments run with protein purified using the spin-filter, the scale of the transcription reaction described above (2.5.3) was tripled.

For the tripled transcription reactions, Ni^{2+} purification was performed by first taking 90 μl of Ni^{2+}-NTA beads (Qiagen) and washing with 270 μl of buffer A (100 mM sodium phosphate pH 7.8, 2% SDS) three times. The beads were then added to the transcription reaction with 180 μl of buffer A. The beads were incubated with the transcription reaction for three hours at 4ºC. The beads were then washed 4 X 270 μl of 5 mM imidazole in buffer A. The protein was then eluted off the beads with 150 μl of 300 mM imidazole in buffer A. This sample solution (α subunit in buffer A with 300 mM imidazole) was used in a few photolysis experiments. However based on observations discussed in the results section, removal of the imidazole is essential in the success of the photolysis experiments.

Ultra free – MC Millipore centrifugal filter devices (30 kD) (spin-filter) were used to separate protein from imidazole. Spin-filters were blocked with 300 μl of 1 X BSA in buffer B (100 mM sodium phosphate pH 7.4, 2% SDS) for 2 hours at room temperature before protein was applied to the filter. The filter was then washed three times with 300

□l of buffer B. Eluted protein from Ni^{2+} bead purification (75 □l to 150 □l) was diluted with 200 □l of buffer B and then applied to the spin-filter. The sample was diluted three times with 200 □l of buffer B. The sample was then centrifuged down to the desired volume, usually between 40-80 □l depending how much eluted protein was added to the spin-filter. The purified sample was removed from the spin-filter and used in photolysis experiments.

2.5.5 *In vivo* suppression of NBSeOH:

The site-directed mutagenesis of Shaker B TAG mutants, gene construction, and synthesis of suppressor tRNA, and ligation of aminoacyl-dCA to tRNA have been described previously (1, 29, 30). Constructs used in Pam England's ShB suppression experiments were used for NBSeOH *in vivo* suppression experiments (1). Plasmid DNAs were linearized with Not1, and mRNA was transcribed using the Ambion T7 mMESSAGE mMACHINE kit.

Oocytes from *Xenopus laevis* were isolated and treated according to published procedures (31, 45). Each oocyte was microinjected (50 nl) with a 1:1 mixture of mRNA for the ShB L47TAG construct (0.04 ng/nl) and tRNA (1 □g/□l). The tRNA was either tRNA amino-acylated to nitroveratryloxycarbonyl (NVOC) protected leucine (Leu), N-pent-4-enoyl protected 2-nitrophenylglycine (Npg), or unprotected NBSeOH. In addition, un-acylated tRNA (dCA control) was also injected with ShB L47TAG mRNA. The tRNA-Leu and tRNA-Npg were deprotected prior to injection according to published procedures (1, 31). In most experiments, little to no current was seen with dCA controls.

Electrophysiology

Whole cell currents from oocytes were measured using a Geneclamp 500 amplifier (Axon Instruments) in the 2-electrode voltage-clamp configuration. Current and voltage electrodes were filled with 3 M KCl to yield resistances ranging from 0.5 to 1 M . The holding potential was either –80 mV or –40 mV. The currents from ShB expressing oocytes were measured during depolarizing jumps from the holding potential to various test potentials between –60 mV to +60 mV in 20 mV jumps. Leakage currents were subtracted using P/4 procedure (1). The oocytes were continuously bathed in ND-96 calcium free bath solution (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES (pH 7.5)).

The oocytes were irradiated on the electrophysiology rig. The apparatus used to irradiate samples has been reported previously (46). Light from 300-W Hg arc lamp was filtered through a Schott UG11 filter to provide 300-350 nm light. The light was focused through 50 mm quartz lens (Oriel) onto a liquid light guide (Oriel, 1 m long, 3mm diameter) connected to the recording chamber. The end of the light guide contacted a Pyrex coverslip placed at the bottom of the recording chamber, upon which the ooctye rested.

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