

## CHAPTER 6: New Approaches to Photochemical Cleavage of Peptide and Protein Backbones\*

### 6.1 ABSTRACT

A strategy for photochemical cleavage of peptide and protein backbones is described, which is based on a selenide-mediated cleavage of a backbone ester moiety. Studies in model systems establish the viability of the chemistry and suggest the method could be a valuable tool for chemical biology studies of proteins. Also described are two alternative strategies based on a “caged” aniline and the (2-nitrophenyl)ethyl (NPE) protecting group.

### 6.2 INTRODUCTION

We describe here new strategies for photochemical cleavage of peptide and protein backbones and model studies intended to evaluate the viability of the novel chemistry involved. Strategies for preparing photoresponsive biomolecules are finding increasing use in chemical biology.<sup>1,2</sup> Some time ago we introduced a strategy for photochemically initiating backbone cleavage of a protein, employing the unnatural amino acid 2-nitrophenylglycine (Npg, **Figure 6.1**).<sup>3</sup> After incorporation of Npg into a protein or peptide, the well-known *o*-nitrobenzyl “deprotection” of the peptide bond nitrogen results in cleavage of the protein backbone, a site-specific, nitrobenzyl-induced, photochemical proteolysis (SNIPP). Other strategies for photochemical cleavage of protein and peptide

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\* This work was done in collaboration with Dr. Amy L. Eastwood and Dr. Niki M. Zacharias and is adapted from: Eastwood, A. L.; Blum, A. P.; Zacharias, N. M.; Dougherty, D. A., A selenide-based approach to photochemical cleavage of peptide and protein backbones at engineered backbone esters. *The Journal of Organic Chemistry* **2009**, 74, (23), 9241-44. Copyright 2009 American Chemical Society.

backbones have recently appeared. Imperiali and Kron have both employed *o*-nitro- $\beta$ -phenylalanine as a linker between two protein or peptide fragments,<sup>4, 5</sup> while Muir has employed an expanded nitrobenzyl linker.<sup>6</sup> Otaka and co-workers introduced a novel system based on a nitrobenzyl-caged phenol and the “trimethyl lock” motif, which promoted intramolecular cleavage of the backbone amide after the phenol was decaged.<sup>7</sup> Schultz and co-workers demonstrated a novel cleavage mediated by 2-nitrophenylalanine.<sup>8</sup>

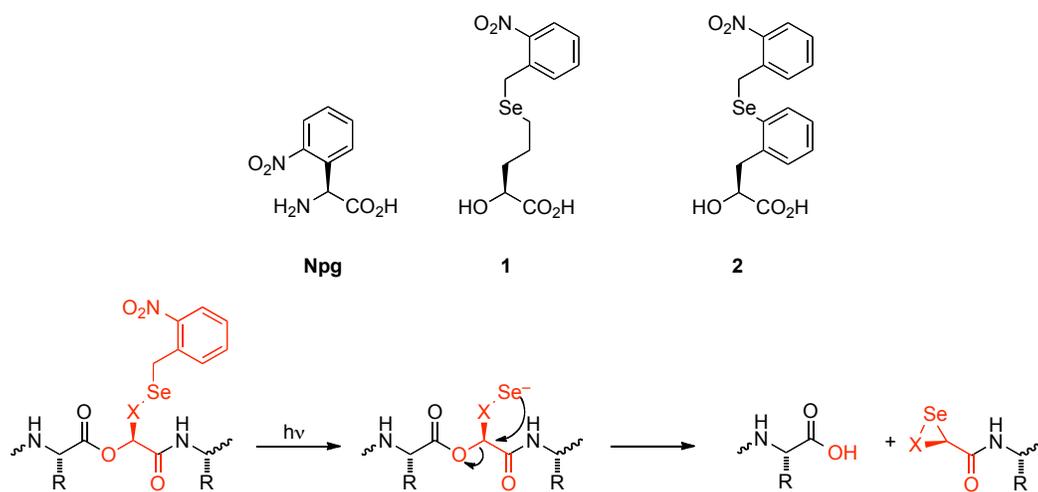
All of these strategies have advantages and disadvantages. The Npg approach has been employed in several contexts<sup>3, 9, 10</sup> and is compatible with both solid phase peptide synthesis (SPPS) and *in vivo* incorporation into full proteins via nonsense suppression. However, the photochemical efficiency of the cleavage is not high, with a ~50% cleavage yield after four hours of photolysis.<sup>3</sup> Also, the incorporation into proteins of the relatively crowded,  $\beta$ -branched residue is often not efficient. Similarly low photoefficiency is seen with 2-nitrophenylalanine.<sup>8</sup> The other strategies can show more efficient cleavage of peptides but are not compatible with *in vivo* incorporation into proteins.

Described herein are alternative strategies for the photochemical cleavage of peptide and protein backbones. The first strategy is based on a caged selenide. Model studies establish that this strategy is effective, with up to 72% backbone cleavage of depsipeptides. Other strategies are also described which are based on a nitroveratryloxycarbonyl (NVOC)-protected aniline and the photochemistry of (2-nitrophenyl)ethyl (NPE) derivatives.

## 6.3 RESULTS

### 6.3.1 Caged selenide strategy

In our first strategy the key cleavage reaction is based on a novel, intramolecular  $S_N2$  reaction, shown schematically in **Figure 6.1**. Selenide is one of the most potent nucleophiles known, and at physiological pH a selenol ( $pK_a \sim 5-6$ ) should be predominantly in the selenide form. The essential reaction,  $S_N2$  cleavage of an ester carbon with a carboxylate as the leaving group, has ample precedent.<sup>11-15</sup> Formation of the ester leaving group requires incorporation of an  $\alpha$ -hydroxy acid (rather than an  $\alpha$ -amino acid), but such backbone esters can be efficiently incorporated into peptides by SPPS<sup>16, 17</sup> and into proteins by nonsense suppression.<sup>10, 18-22</sup> In addition, selenium-containing natural amino acids such as selenocysteine and selenomethionine are well-known to be efficiently incorporated into proteins. Finally, “caging” the selenide with an *o*-nitrobenzyl group allows the process to be initiated photochemically.



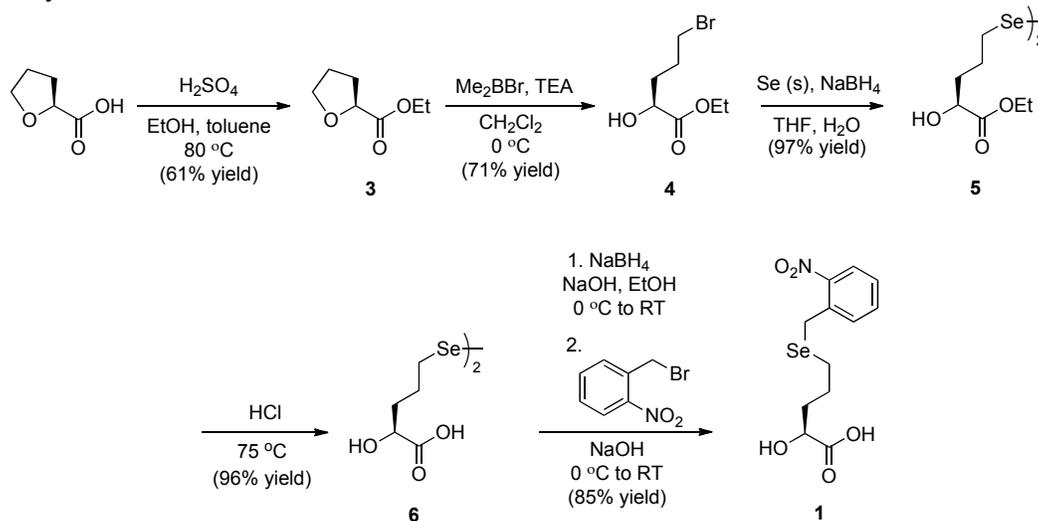
**Figure 6.1.** Npg and the second-generation SNIPP unnatural  $\alpha$ -hydroxy acids, **1** and **2**.

The process proposed in **Figure 6.1**, however, raises many questions. Like all  $S_N2$  reactions, the selenide-induced ester displacement is sensitive to steric effects. As such, the reaction is typically applied to methyl esters, although under optimal conditions and with heating, ethyl, benzyl, and even isopropyl esters along with many lactones are reactive. Certainly, the  $\alpha$ -carbon of the  $\alpha$ -hydroxy acid that is incorporated will be sterically crowded. The question is whether the intramolecularity of the process, perhaps aided by the inductive effect of the neighboring amide carbonyl, will overcome the steric burden. Caged selenides are not common, and so there is the question of the efficiency of the photochemical step. In addition, selenides are sensitive to oxidation, more so for aliphatic than aromatic (selenophenol) derivatives. On the other hand, an aliphatic selenide might be expected to be the stronger nucleophile, but an aryl selenide has fewer rotatable bonds that need to be restricted in the cyclization reaction.

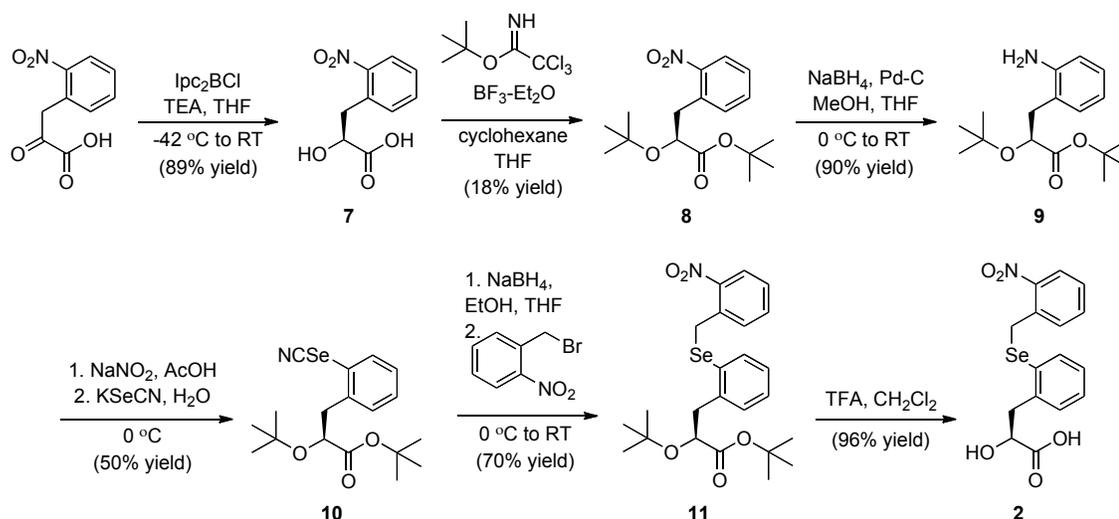
Given these chemical uncertainties, it seemed prudent to first evaluate the viability of the chemistry proposed in **Figure 6.1** before proceeding with chemical biology studies. In the present work we evaluate two structures that are meant to provide such a test. We describe the synthesis and characterization of aliphatic (**1**) and aromatic (**2**) variants of the design, along with mechanistic characterization.

Synthesis of enantiopure **1** (**Scheme 6.1**), as devised and executed by Dr. Amy L. Eastwood<sup>23</sup> and Dr. Niki M. Zacharias,<sup>24</sup> began with conversion of *S*-(-)-tetrahydro-2-furoic acid to the ring-opened bromide **4** as previously described.<sup>25, 26</sup> Conversion to the diselenide, and ester hydrolysis then produced **6**.<sup>27, 28</sup> Acid diselenide **6** was directly reduced with sodium borohydride and the product alkylated with *o*-nitrobenzyl bromide to give the target compound **1**.<sup>29</sup>

**Scheme 6.1.** Synthesis of selenide  $\alpha$ -hydroxy acid **1**. This route was devised and executed by Dr. Amy L. Eastwood<sup>23</sup> and Dr. Niki M. Zacharias.<sup>24</sup>



**Scheme 6.2.** Synthesis of selenide  $\alpha$ -hydroxy acid **2**.

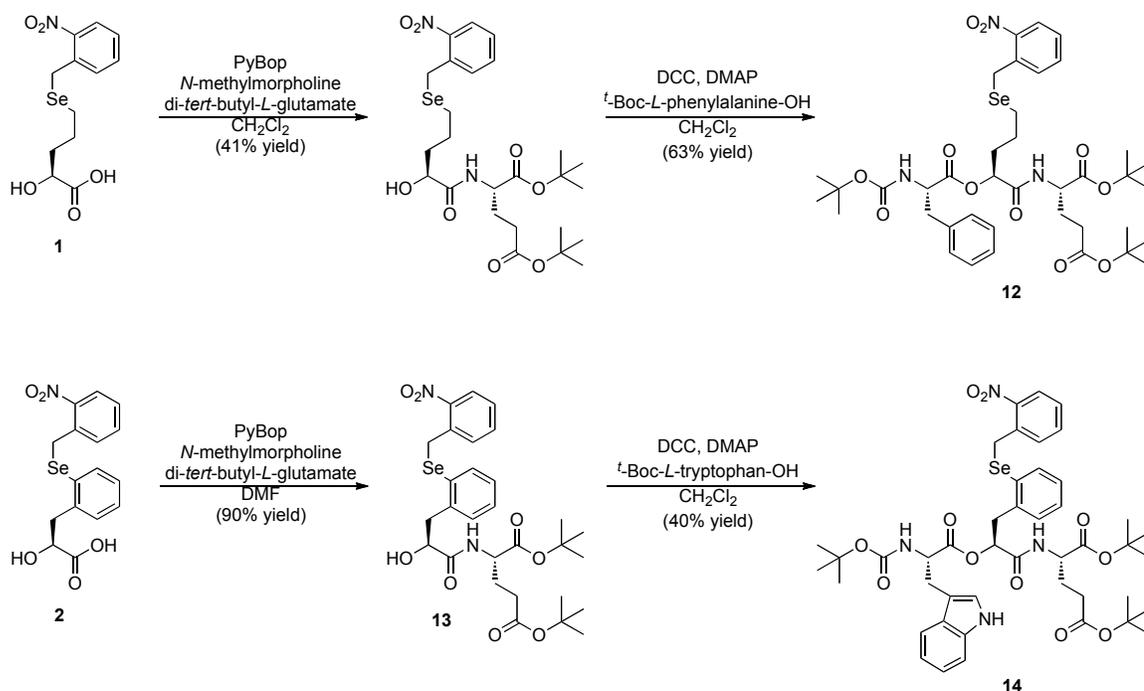


The synthesis of **2** (Scheme 6.2) began with the known reduction of 2-nitrophenylpyruvic acid by (+)- $\beta$ -chlorodiisopinocampheylborane (“Ipc<sub>2</sub>BCl”) to yield **7** in 94% ee.<sup>30</sup> The selenocyanate was prepared by a modification of the standard sequence, and the nitrobenzyl group was introduced by reductive alkylation. The bulky *t*-butyl protecting groups were installed to discourage intramolecular cyclization, which was seen when **7** was subjected to reducing conditions, as well as to improve the

solubility and ease of purification of subsequent compounds in the sequence. Alternative synthetic routes were also attempted and are described in Appendix 3.

To evaluate whether the proposed cleavage mechanism was viable, studies in model systems were performed. Depsipeptides **12** and **14** were chosen for synthetic accessibility and because they introduce a UV chromophore into the carboxylate cleavage product. They were prepared through standard solution-phase coupling procedures, employing PyBop/*N*-methylmorpholine and DCC/DMAP for the hydroxy-peptide- and depsipeptide-forming reactions, respectively (**Scheme 6.3**).

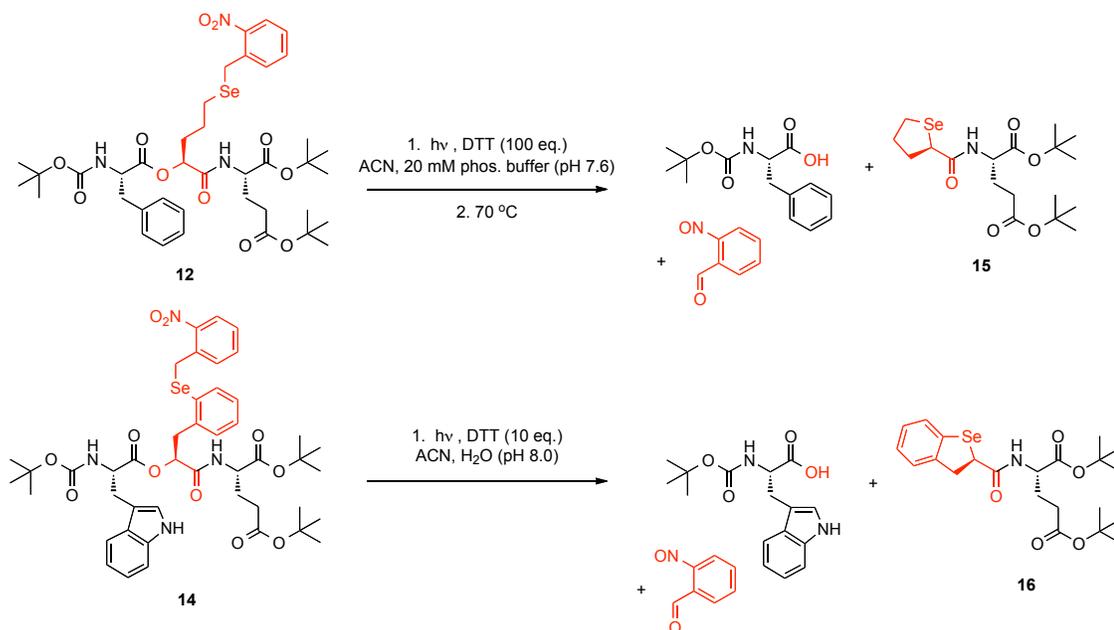
**Scheme 6.3.** Synthesis of depsipeptides **12** and **14**. Depsipeptide **12** was prepared by Dr. Amy L. Eastwood.<sup>23</sup>



Mass spectrometric analysis of initial photolysis studies indicated the formation of the mechanistically revealing selenacyclopentane (**15**, **16**) and the appropriate carboxylic acid, but also suggested several complicating side reactions. Both **12** and **14** produced product *m/z* ratios consistent with the dimer of the deprotected selenide, including the

characteristic isotope pattern for a structure with two selenium atoms. In addition, the aliphatic variant **12** showed  $m/z$  ratios consistent with a depsipeptide containing dehydronorvaline (*i.e.*, an allyl side chain), presumably formed by elimination of the selenoxide produced by air oxidation. These undesirable reactions could be suppressed by running the photolyses in the presence of excess dithiothreitol (DTT),<sup>31</sup> which was expected to discourage both dimerization and oxidation.

**Scheme 6.4.** Depsipeptide cleavage reactions.



For aryl selenide **14** an unanticipated side product, identified by mass spectrometry, was the depsipeptide in which the original nitrobenzylselenenyl group had been replaced by a hydrogen atom. While this work was in progress, Kitahara and co-workers reported that short wavelength photolysis of arylalkyl selenides can lead to C-Se bond cleavage.<sup>32</sup> As suggested by that study, we found that changing the photolysis wavelength from >300 nm (pyrex filter) to >330 nm (uranium glass filter) eliminated this side reaction.

Of course, the novel process of **Figure 6.1** cannot be considered validated without unambiguous confirmation that the selenacyclopentanes **15** and **16** were formed. Using the knowledge gained from the preliminary studies, preparative-scale photolyses were performed (**Scheme 6.4**). For both depsipeptides the selenacyclopentane product could be isolated after irradiation, providing clear support for the proposed scheme; mass spectrometry and NMR spectroscopy confirm product identities. We find that cleavage is more efficient with the aryl selenide (**14**) than the alkyl (**12**), in that isolated yields of the selenacyclopentane are consistently higher (72% vs. 29%). In addition, obtaining significant yields of the selenacyclopentane from the aliphatic system (**12**) required heating the photolysis mixture to ~70 °C, which was not necessary in the case of aryl selenide **14** under optimal photolysis conditions. These observations, coupled with the lack of complications due to olefin formation, suggest that aryl selenide **2** may be the better system to incorporate for subsequent protein and peptide studies.

#### *6.3.1.1 Chemical biology studies of proteolysis by 1 and 2*

In preparation for nonsense suppression experiments, the key  $\alpha$ -hydroxy acids **1** and **2** were each activated as a cyanomethyl ester and subsequently transesterified by published protocols<sup>33</sup> to yield acylated suppressor tRNA as needed for the following experiments.

#### *6.3.1.2 In vitro studies of proteolysis by 1 and 2*

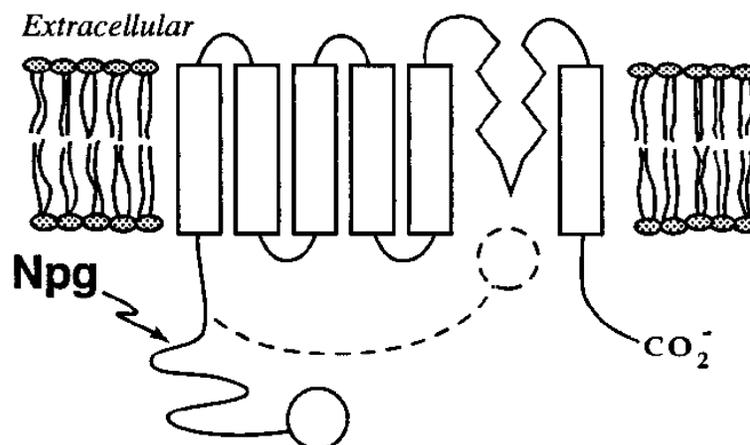
Studies were conducted to determine whether **1** or **2** could promote proteolysis of a full-length protein *in vitro*. These studies used *in vitro* nonsense suppression methodology to incorporate **1** and **2** into the  $\alpha 1$  subunit of the muscle-type nicotinic acetylcholine receptor (nAChR) at residue Met243 (located in the M2 transmembrane helix). In these

studies, the  $\alpha 1$  subunit of the nAChR contains a hemmagglutinin epitope (in the M3-M4 cytoplasmic loop) to facilitate Western blotting. It was envisioned that cleavage of the nAChR protein backbone at Met243 would yield cleavage fragments that are sufficiently different in molecular weight from the full-length protein to be easily separated by polyacrylamide gel electrophoresis (PAGE) and subsequently visualized by Western blotting. We chose rabbit reticulocyte and wheat germ lysate as our *in vitro* translation systems. Rabbit reticulocyte is generally the preferred system for *in vitro* nonsense suppression, because it tends to yield higher quantities of protein than other systems, but the deep red color of this lysate (owing to its high heme content) was anticipated to complicate experiments requiring irradiation with UV light. Wheat germ lysate generally gives lower protein yields, but it is translucent and therefore more amenable to our experiments. Both systems successfully incorporated **1**, but not **2** into the nAChR. While it is unclear why proteins containing **2** did not express, it is likely that the side chain of this unnatural amino acid was too large or otherwise incompatible with the ribosomes of these translation systems. Proteins containing **1** were susceptible to base (concentrated  $\text{NH}_4\text{OH}$ ) cleavage of the backbone ester, but irradiation of the samples never resulted in the appearance of cleavage products when visualized by Western blotting. Attempts to optimize photolysis conditions (including heating the solutions before or after irradiation and increasing the pH of the media) were unsuccessful. Additional *in vitro* studies (also unsuccessful) are described in the theses of Dr. Amy L Eastwood<sup>23</sup> and Dr. Niki M. Zacharias.<sup>24</sup>

### 6.3.1.3 Nonsense suppression experiments with **1** and **2** in *Xenopus* oocytes

Nonsense suppression experiments were also conducted in *Xenopus* oocytes. These studies were based on experiments designed to evaluate the effectiveness of Npg, the original unnatural amino acid shown to promote photochemical cleavage of protein backbones. In these experiments, Npg was expressed in the N-terminal domain of the *Drosophila* Shaker B K<sup>+</sup> channel at residues Leu47 and Pro64.<sup>3</sup>

The Shaker B (ShB) channel is a voltage-gated ion channel that is comprised of four identical subunits. Each subunit consists of six transmembrane regions, a short intracellular C-terminus and a long intracellular N-terminus (**Figure 6.2**). The pore loop resides between the fifth and sixth transmembrane regions. The first twenty amino acids of the N-terminus form a structural domain or “ball” that is known to inactivate the channel on a millisecond timescale by plugging the channel’s pore (through “ball and chain” or “N-type” inactivation).<sup>34</sup> The ball domain is tethered to the remainder of the protein by a “chain” sequence of ~60 amino acids. Channel inactivation occurs when any of the four balls of the homotetrameric protein plug the channel pore. Deletion of the ball and chain regions (residues 6-46) results in the well-characterized ShakerIR (ShIR; where IR stands for “inactivation domain removed”) channel that does not inactivate on a millisecond timescale.<sup>35-37</sup>



**Figure 6.2.** Depiction of the topology of the Shaker B  $K^+$  channel and the location of sites used to incorporate Npg. Figure is adapted from England *et al.*<sup>3</sup>

Npg was inserted at Leu47 and Pro64 in the N-terminal ball and chain domain of ShB.<sup>3</sup> Irradiation of the mutant channels lead to cleavage of the inactivation ball (essentially converting the ShB protein into ShIR) and a corresponding reduction in the channel inactivation as measured by two-electrode voltage-clamp electrophysiology. Thus cleavage of the inactivation ball of the ShB protein provides a convenient phenotype for evaluating the effectiveness of **1** and **2** in promoting protein backbone cleavage.

Negative control experiments led us to question the fidelity of nonsense suppression experiments at Leu47 and Pro64 using the amber THG73 suppressor tRNA. These controls included injection of THG73 without an appended amino acid to look for misacylation or “reaminoacylation” – a situation in which the suppressor tRNA is charged with a natural amino acid by an endogenous aminoacyl-tRNA synthetase, which enables incorporation of the natural amino acid at the mutation site. From these controls it was clear that misacylation was likely to be occurring, suggesting that natural amino acids might be incorporated at Leu47 and Pro64 during nonsense suppression

experiments. For this reason, we turned to frameshift nonsense suppression, because the suppressor tRNA used in this methodology is more orthogonal with the *Xenopus* oocyte translational system.<sup>38, 39</sup>

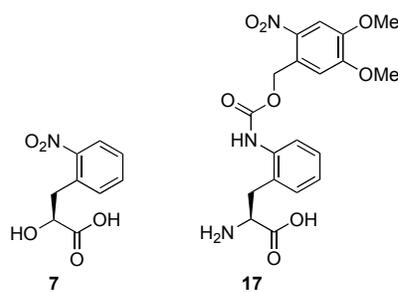
Compounds **1** and **2** were successfully incorporated into the ShB protein expressed in *Xenopus* oocytes using frameshift nonsense suppression methodology as indicated by the larger currents (10-fold higher) seen for suppression with the unnatural residues relative to control (misacylation) experiments. Unfortunately, irradiation of ShB proteins expressing **1** or **2** did not give the ShIR phenotype, suggesting that, unlike Npg,  $\alpha$ -hydroxy acids **1** and **2** could not promote backbone cleavage in these systems. Several attempts were made to optimize the conditions, including increasing the pH of the ND96 recording solution to ensure deprotonation of the selenol (to yield a the more reactive selenide), prolonged irradiation times (5 min to several hrs) with different light sources (a 1000 W Hg/Xe arc lamp and a 288 W Hg lamp), but we were never able to see evidence of backbone cleavage by either compound. Thus, despite their success in cleaving protein backbones of depsipeptide model systems, **1** and **2** have not been shown to be capable of promoting backbone cleavage *in vitro* or *in vivo*.

### ***6.3.2 Strategies based on a photocaged aniline and the chemistry of the NPE protecting group***

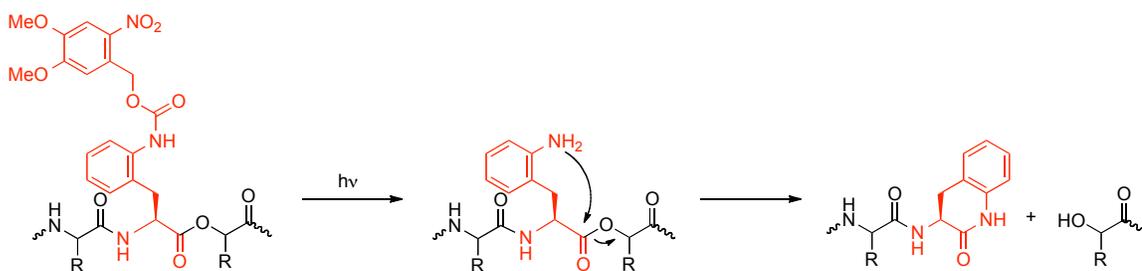
Two additional strategies for cleaving peptide and protein backbones were also considered. The first was based on an nitroveratryloxycarbonyl (NVOC) caged aniline (**17**) in which the key cleavage reaction is an intramolecular cyclization to afford a  $\delta$ -lactam (**Figure 6.3**). Incorporation of an  $\alpha$ -hydroxy acid at the *i+1* residue yields a strong alkoxide leaving group. The motivation for this strategy came during the synthesis of **2**

in which direct reduction of the precursor compound **7** resulted in intramolecular cyclization (discussed in further detail in Appendix 3).

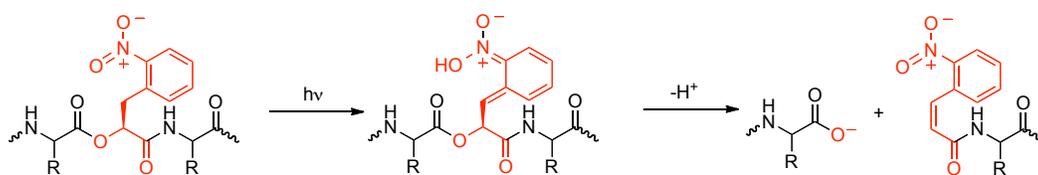
The second strategy (**Figure 6.4**) is based the photochemistry of the (2-nitrophenyl)ethyl (NPE) protecting group,<sup>40, 41</sup> in which incorporation of the  $\alpha$ -hydroxy acid analog of 2-nitrophenylalanine (compound



**7**) into a protein creates an NPE-protected backbone ester. Irradiation of the protein results in “deprotection” of the ester and cleavage of the protein backbone. This produces two protein fragments— one with a carboxy terminus and another, which is essentially an  $\alpha,\beta$ - unsaturated ketone. We appreciate that the latter product could complicate our studies by serving as a cross-linking agent, but we were still interested in seeing whether this strategy could promote backbone cleavage *in vivo* especially given that we had already synthesized **7** for other purposes (**Scheme 6.2**).



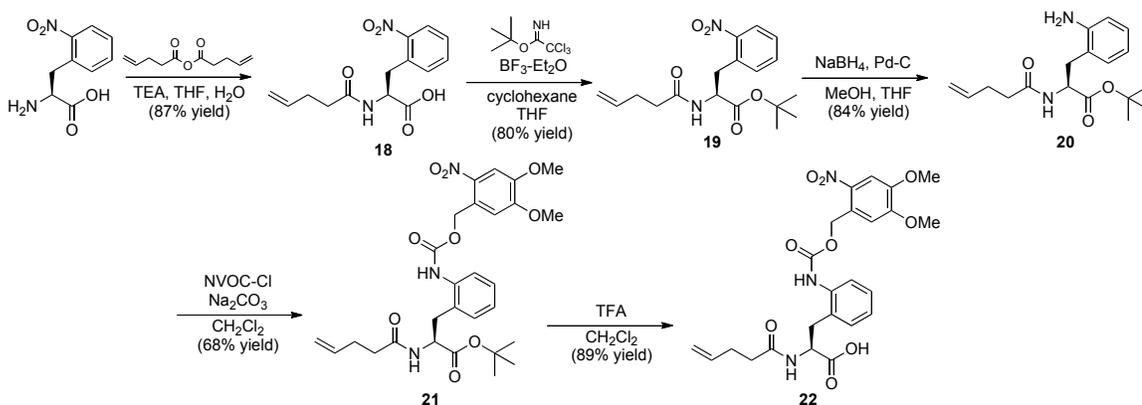
**Figure 6.3.** Proposed photochemical cleavage strategy using caged aniline **17**.



**Figure 6.4.** Proposed photochemical cleavage strategy using  $\alpha$ -hydroxy acid **7**.

The synthesis of the *N*-pent-4-enoyl (4PO) derivative of **17** (**Scheme 6.5**) began with the protection of the  $\alpha$ -amino group of *L*-2-nitrophenylalanine as the *N*-pent-4-enoyl (4PO) derivative (cleavable by treatment with  $I_2$ )<sup>3, 42, 43</sup> and the carboxylic acid as the *t*-butyl ester (to prevent intramolecular cyclization) using standard protocols. Afterward, the nitro group was reduced following the protocol used in **Scheme 6.2**, and the resulting aniline was caged with a photocleavable nitroveratryloxycarbonyl (NVOC) group. Removal of the *t*-butyl protecting group gave the desired product **22**. In preparation for nonsense suppression experiments, **7** and **22** were activated as cyanomethyl esters and subsequently transesterified according to published protocols<sup>33</sup> to yield acylated TQOps' (opal suppressor tRNA for TGA stop codon), THG73 (amber suppressor tRNA for TAG stop codon), and YFaFs (suppressor tRNA for GGGT frameshift codon) suppressor tRNA.

**Scheme 6.5.** Synthesis of the *N*-pent-4-enoyl (4PO) derivative of **17**.



### 6.3.2.1 Nonsense suppression experiments with **7** and **17** in *Xenopus oocytes*

Given that the caged aniline-based strategy described in **Figure 6.3** requires the incorporation of two adjacent unnatural amino acids (**17** and an  $\alpha$ -hydroxy acid in the  $i+1$

position), it seemed prudent to find an appropriately tolerant site in the N-terminal region of the ShB protein (in order to make use of the ShB to ShIR cleavage phenotype).

Several combinations of stop codons and the four-base codon were used in initial screens of Pro64 (a site that Npg was incorporated at) and the *i+1* residue Lys65 (**Table 6.1**). Pro and Vah (valine,  $\alpha$ -hydroxy) were used as indicators of the maximal current values that could be expected for nonsense suppression experiments with each codon combination. Unacylated suppressor tRNA (represented in **Table 6.1** as a dash (-)) was also used as a misacylation control. As shown in **Table 6.1**, only the Pro65TGA/Lys65TAG and Pro65GGGT/Lys65TAG combinations gave current in these experiments, and no current was seen in misacylation control experiments after a 24 hr of incubation period. To our knowledge, this is the first example of double nonsense suppression at sites that are adjacent in sequence.

**Table 6.1.** Current ( $I_{\max}$ ) obtained for control studies of Pro64 and Lys65 in ShB using different codon combinations. “n.r.” stands for no response. A dash (-) represents a misacylation control experiment. “WT” is the wild-type ShB protein.

Mutation	Residue at Pro64	Residue at Lys65	$I_{\max}$ ( $\mu$ A)	Incubation Time (hr)
WT			$42 \pm 5.6$	12
ShB(Pro64TGA)/(Lys65TAG)	-	Vah	n.r.	24
	Pro	-	n.r.	24
	Pro	Vah	$8.3 \pm 1.1$	24
ShB(Pro64GGGT)/(Lys65TAG)	-	Vah	n.r.	24
	Pro	-	n.r.	24
	Pro	Vah	$5.5 \pm 0.91$	24
ShB(Pro64GGGT)/(Lys65TGA)	-	-	n.r.	24
	Pro	Vah	n.r.	24
ShB(Pro64TAG)/(Lys65TGA)	-	-	n.r.	24
	Pro	Vah	n.r.	24

Given these results, we then incorporated **7** and **17** at Pro64 using the TAG stop codon or GGGT frameshift codon. After 48 hrs of incubation, current was seen for proteins injected with **7** and **17**, but the  $I_{\max}$  values were on the order of what was seen for misacylation control experiments (**Table 6.2**), which questioned the fidelity of these

experiments. Irradiation of oocytes injected with **7** and **17** had no effect on the inactivation of the ShB currents, suggesting that these residues cannot cleave the peptide backbone or that **7** and **17** did not actually incorporate into the protein and the currents observed are the result of misacylation of the suppressor tRNA (incorporation of a natural amino acid at the Pro64 site).

**Table 6.2.** Current ( $I_{\max}$ ) obtained for studies with **7** and **17** at Pro64 in ShB. “n.r” stands for no response. A dash (-) represents a misacylation control experiment. Note that no current was seen after 24 hours for **7** or **17**. “WT” is the wild-type ShB protein.

Mutation	Residue at Pro64	$I_{\max}$ ( $\mu\text{A}$ )	Incubation Time (hr)
WT		$52 \pm 10$	12
ShB(Pro64TGA)	-	$8.7 \pm 2$	48
	<b>7</b>	$0.99 \pm 1$	48
ShB(Pro64GGGT)	<b>17</b>	n.r.	48
	-	$4.7 \pm 0.7$	48
	<b>7</b>	$8.3 \pm 2$	48
	<b>17</b>	$12 \pm 2$	48

We then repeated these experiments with **17** at Pro64 and Vah at Lys65 (to give a better leaving group for the cleavage reaction). Unfortunately, no current was seen for these experiments for reasons that we do not presently understand. Future work could revisit these studies by trying different residues in the ShB protein or a different  $\alpha$ -hydroxy acid at the  $i+1$  residue.

During the course of our studies, Schultz and co-workers reported a photochemical cleavage strategy that is nearly identical to **Figure 6.4** and is based on the amino acid derivative of **7** (2-nitrophenylalanine).<sup>8</sup> They showed that 2-nitrophenylalanine could promote backbone cleavage of model peptides and also of a model protein, T4 lysozyme, expressed in *E. coli*, albeit with only 30% photochemical cleavage efficiency of the latter system. These findings are certainly encouraging (if not

overlapping) and so it would be interesting to revisit the studies of **7** to determine the origin of our photolysis problems.

## 6.4 DISCUSSION

In summary, we first described novel chemistry in which a photochemically-liberated selenide undergoes an intramolecular  $S_N2$  reaction, cleaving an ester that results from the incorporation of the  $\alpha$ -hydroxy acids **1** or **2** into a peptide. These studies suggest that for chemical biology applications, aryl selenide **2** is the preferred substrate. Two side reactions have been discovered that must be considered in possible chemical biology applications: dimerization and oxidation. For studies involving peptides prepared by SPPS, adding DTT and controlling the concentration might be appropriate. For *in vivo* nonsense suppression expression experiments, where protein concentrations are typically low, dimerization may be less likely. In addition, the reducing conditions inside cells likely will discourage dimerization and oxidation. Unfortunately, our initial efforts to see backbone cleavage by **1** or **2** *in vivo* and *in vitro* have been unsuccessful, but additional studies should be conducted to determine whether other applications of this methodology are possible.

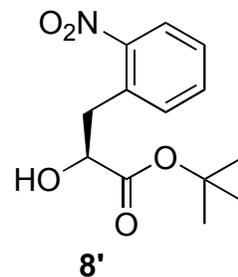
We also described two alternative strategies for photochemical protein cleavage that were inspired by chemistry we encountered during our synthesis of **2**. These strategies are based on (1) intramolecular cyclization and backbone cleavage by a photochemically-liberated aniline nucleophile and (2) the photochemistry of (2-nitrophenyl)ethyl derivatives. These new unnatural amino acids were successfully synthesized, but photolysis studies in proteins expressed in *Xenopus* oocytes have been unsuccessful thus far.

## 6.5 EXPERIMENTAL SECTION

**Chemical synthesis.** All reactions were performed at ambient temperature and pressure unless otherwise noted. All reactions involving potentially air-sensitive compounds were conducted under an inert atmosphere using Schlenk techniques. Solvents were purified by passage through alumina. Unless otherwise noted, all chemicals and reagents were used as received without further purification. Compounds **3**,<sup>25</sup> **4**,<sup>26</sup> and **7**<sup>30</sup> were prepared according to published protocols. The syntheses of **1** and **12** were performed by Dr. Amy L. Eastwood<sup>23</sup> and Dr. Niki M. Zacharias<sup>24</sup> and are described in their respective theses and also in Eastwood *et al.*<sup>44</sup> Flash chromatography was performed with EMD silica gel 60 (particle size 0.040-0.063 mm). Thin-layer chromatography (TLC) was performed using EMD silica gel 60 F<sub>254</sub> precoated plates (0.25 mm) and visualized by UV light, potassium permanganate, ceric ammonium molybdate, or ninhydrin. Nuclear magnetic resonance spectroscopy (NMR) was performed on either a Varian Mercury 300 or a Varian Inova 500 instrument and spectra resonances are assigned relative to Me<sub>4</sub>Si ( $\delta$  0.0) or CD<sub>3</sub>OD ( $\delta$  3.31 for <sup>1</sup>H NMR and  $\delta$  49.1 for <sup>13</sup>C NMR). Data for <sup>1</sup>H NMR spectra are reported as follows: chemical shift ( $\delta$  ppm), integration, multiplicity, and coupling constant (Hz). Data for <sup>13</sup>C NMR spectra are reported as chemical shift ( $\delta$  ppm). High-resolution mass spectrometry (HRMS) spectra were obtained from the Caltech Mass Spectrometry Lab. Electrospray ionization mass spectrometry (ESI-MS) used to analyze the proteolysis reactions was performed using an LCQ Classic ion trap (ThermoFinnigan) in direct infusion mode. A uranium glass absorption sleeve was prepared by the Caltech Glassblowing Shop.

**Synthesis of di-*tert*-butyl-protected nitrophenylalanine derivative (**8**) and *tert*-butyl-protected nitrophenylalanine derivative (where the secondary alcohol is not protected) (**8'**).**

$\alpha$ -Hydroxy acid **7** (0.200 g, 0.947 mmol, 1 eq) was placed in a 2-neck round-bottom flask under Ar (g) and dissolved in THF (3 mL) and cyclohexane (3.5 mL). A solution of *tert*-butyl-2,2,2-trichloroacetimidate (0.678 mL, 3.79 mmol, 4 eq) in cyclohexane (3.5 mL) was added simultaneously with boron



trifluoride diethyletherate (0.041 mL, 0.33 mmol, 0.35 eq). The reaction stirred for 45 minutes until it was quenched with saturated NaHCO<sub>3</sub> (aq). The organics were extracted with Et<sub>2</sub>O (3 $\times$ ), washed with brine, dried over MgSO<sub>4</sub>, and concentrated. The resulting white sludge was suspended in hexanes, filtered, and then purified by flash column chromatography (15% EtOAc in hexanes) to afford two fractions: compound **8** (0.0550 g, 0.170 mmol, 18% yield) and compound **8'** (0.101 g, 0.379 mmol, 40% yield) as clear oils.  $R_f$  of **8** = 0.71 (30% EtOAc in hexanes); <sup>1</sup>H NMR of **8** (300 MHz, CDCl<sub>3</sub>, 298 K)  $\delta$  7.91 (1H, d,  $J$  = 8.0 Hz), 7.50 (1H, m), 7.37 (2H, m), 4.14 (1H, dd,  $J$  = 8.9, 4.9 Hz), 3.29 (1H, dd,  $J$  = 13.2, 4.9 Hz), 3.13 (1H, dd,  $J$  = 13.2, 9.1 Hz) 1.39 (9H, s), 0.95 (9H, s); <sup>13</sup>C NMR of **8** (75 MHz, CDCl<sub>3</sub>, 298 K)  $\delta$  172.9, 149.9, 134.5, 132.9, 132.6, 127.9, 124.7, 81.2, 75.2, 71.8, 37.7, 28.0, 27.6; HRMS (FAB) of **8**  $m/z$  calc'd for C<sub>17</sub>H<sub>26</sub>NO<sub>5</sub> [M+H]: 324.1811, found 324.1820.  $R_f$  of **8'** = 0.44 (30% EtOAc in hexanes); <sup>1</sup>H NMR of **8'** (300 MHz, CDCl<sub>3</sub>, 298 K)  $\delta$  7.90 (1H, d,  $J$  = 8.1 Hz), 7.51 (1H, m), 7.40 (2H, m), 4.34 (1H, dd,  $J$  = 8.1, 4.1 Hz), 3.48 (1H, dd,  $J$  = 13.8, 4.3 Hz), 3.13 (1H, dd,  $J$  = 13.9, 8.3 Hz), 1.42 (9H, s); <sup>13</sup>C NMR of **8'** (75 MHz, CDCl<sub>3</sub>, 298 K)  $\delta$  173.5, 150.1, 133.4, 133.0, 132.4, 128.1, 125.0, 83.3, 70.7, 37.5, 28.1; HRMS (FAB) of **8'**  $m/z$  calc'd for C<sub>13</sub>H<sub>18</sub>NO<sub>5</sub>

[M+H]: 268.1185, found 268.1193. An enantiomeric excess of 94% was obtained for **8'** by analytical chiral HPLC analysis using a Chiralcel OD-H column (4.6 mm × 25 cm) from Daicel Chemical Industries, Ltd. with 2% isopropyl alcohol in hexanes.

**Synthesis of aniline (9).** Di-*tert*-butyl-protected nitrophenylalanine derivative **8** (0.606 g, 1.87 mmol, 1 eq) was placed in a 2-neck round-bottom flask under Ar (g) and dissolved in THF (30 mL) and MeOH (15 mL) at 0 °C. To this was added 10 wt % palladium on activated carbon (0.050 g) and sodium borohydride (0.141 g, 3.74 mmol, 2 eq). The reaction was followed by TLC using ninhydrin staining. The solution was stirred for 40 minutes and was then quenched with water and filtered through a pad of Celite™. The filtrate was extracted with Et<sub>2</sub>O (3×), washed with brine, dried over MgSO<sub>4</sub>, and concentrated to afford a pale yellow oil. The resulting liquid was purified by flash column chromatography (15% EtOAc in hexanes) to afford aniline **9** as a pale yellow oil (0.493 g, 1.68 mmol, 90% yield). *R*<sub>f</sub> = 0.59 (30% EtOAc in hexanes); <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>, 298 K) δ 7.27 (1H, d, *J* = 8.9 Hz), 7.17 (1H, t, *J* = 7.4 Hz), 7.04 (1H, d, *J* = 7.3 Hz), 6.83 (1H, t, *J* = 7.3 Hz), 3.99 (1H, dd, *J* = 7.8, 2.3 Hz), 2.96 (1H, dd, *J* = 13.8, 10.4 Hz), 2.68 (1H, dd, *J* = 13.8, 9.1 Hz), 1.46 (9H, s), 0.98 (9H, s); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>, 298 K) δ 173.5, 149.9, 130.3, 127.8, 123.8, 121.0, 114.2, 81.6, 75.6, 74.8, 35.5, 28.0, 27.4; HRMS (ESI) *m/z* calc'd for C<sub>17</sub>H<sub>27</sub>NO<sub>3</sub> [M+H]: 294.2069, found 294.2058.

**Synthesis of selenocyanate (10).** Aniline **9** (0.248 g, 0.845 mmol, 1.0 eq) was placed in a round-bottom flask, dissolved by sonication in AcOH (17 mL), and cooled to 0 °C. To this solution was quickly added 3 M sodium nitrite (0.34 mL, 1.02 mmol, 1.21 eq) via syringe. The solution stirred for 1 hour and was monitored by TLC using ninhydrin

staining. The pH of the solution was then increased to ~6 by the addition of 10 wt % NaOH. To this was added potassium selenocyanate (0.366 mL, 2.54 mmol, 4.2 eq) in H<sub>2</sub>O (36 mL) and the solution stirred for an additional 30 min. The organics were extracted with Et<sub>2</sub>O (3×), washed with brine, filtered through a pad of Celite™, dried over MgSO<sub>4</sub>, and concentrated. The resulting orange solid was purified by flash column chromatography (20% EtOAc in hexanes) to afford selenocyanate **10** as a yellow, non-crystalline solid (0.162 g, 0.423 mmol, 50% yield).  $R_f = 0.83$  (40% EtOAc in hexanes); <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>, 298 K)  $\delta$  7.82 (1H, d,  $J = 7.6$  Hz), 7.26 (3H, m), 3.82 (1H, dd,  $J = 8.7, 4.7$  Hz), 3.06 (2H, m,  $J = 9.48, 4.53$  Hz), 1.46 (9H, s), 0.92 (9H, s); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>, 298 K)  $\delta$  172.4, 139.2, 134.0, 131.1, 129.6, 128.7, 127.6, 105.2, 81.5, 76.2, 73.1, 40.3, 28.0, 27.4; HRMS (ESI)  $m/z$  calc'd for C<sub>18</sub>H<sub>25</sub>NO<sub>3</sub>Se [M+H]: 384.1078, found 384.1073.

**Synthesis of *tert*-butyl-protected arylalkyl selenide (11).** Selenocyanate **10** (0.161 g, 0.421 mmol, 1 eq) was placed in a 2-neck round-bottom flask under Ar (g) and dissolved in THF (7 mL) at 0 °C. To this was added a solution of sodium borohydride (0.019 g, 0.505 mmol, 1.2 eq) in EtOH (1 mL) and the solution stirred for 1 hr. A solution of 2-nitrobenzylbromide (0.118 g, 0.547 mmol, 1.3 eq) in THF (5.5 mL) was then added. After this addition, the solution was allowed to warm to room temperature and was stirred for 3 hours until it was quenched with H<sub>2</sub>O. The organics were extracted with Et<sub>2</sub>O (3×), washed with brine, filtered through a pad of Celite™, dried over MgSO<sub>4</sub>, and concentrated. The resulting solid was purified by flash column chromatography (15% EtOAc in hexanes) to afford *tert*-butyl protected arylalkyl selenide **11** as a yellow, non-crystalline solid (0.145 g, 0.295 g, 70% yield).  $R_f = 0.65$  (30% EtOAc in hexanes); <sup>1</sup>H

NMR (300 MHz, CDCl<sub>3</sub>, 298 K)  $\delta$  8.00 (1H, d,  $J = 7.2$  Hz), 7.45 (1H, d,  $J = 7.1$  Hz), 7.35 (2H, m), 7.21 (2H, m), 7.09 (1H, m), 6.99 (1H, m), 4.33 (2H, s), 4.15 (1H, m), 3.16 (1H, dd,  $J = 9.90, 4.53$  Hz), 2.96 (1H, dd,  $J = 8.24, 5.36$  Hz), 1.43 (9H, s); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>, 298 K)  $\delta$  173.2, 141.2, 136.2, 135.5, 133.2, 132.1, 131.8, 130.9, 128.3, 128.0, 127.6, 125.6, 80.9, 74.9, 72.8, 40.9, 30.0, 28.0, 27.7, 22.4; HRMS (FAB)  $m/z$  calc'd for C<sub>24</sub>H<sub>31</sub>NO<sub>5</sub>Se [M<sup>+</sup>]: 493.1367, found 493.1374.

**Synthesis of arylalkyl selenide  $\alpha$ -hydroxy acid (2).** *tert*-Butyl protected arylalkyl selenide **11** (0.533 g, 1.08 mmol) was placed in a 2-neck round-bottom flask under Ar (g) and dissolved in CH<sub>2</sub>Cl<sub>2</sub> (8 mL). To this was added trifluoroacetic acid (4 mL) and the solution stirred for 16 hours. The reaction mixture was concentrated to afford a yellow-orange solid, which was triturated with ether. The resulting yellow solid was dissolved in EtOAc (3 $\times$ ), rinsed with 1 N HCl, rinsed with H<sub>2</sub>O, and then added to 5% NaHCO<sub>3</sub>. The organic layer (colorless) was discarded, and the pH of the aqueous layer was decreased to pH 2 by the addition of 6 N HCl. The organics were extracted into EtOAc (3 $\times$ ), rinsed with brine, dried over MgSO<sub>4</sub>, and concentrated to afford arylalkyl selenide  $\alpha$ -hydroxy acid **2** as a yellow, non-crystalline solid (0.195 g, 0.512 mmol, 96% yield).  $[\alpha]_D^{24} = -45.3^\circ$  ( $c = 1$ , CHCl<sub>3</sub>); <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>, 298 K)  $\delta$  7.99 (1H, d,  $J = 6.6$  Hz), 7.47 (1H, d,  $J = 7.7$  Hz), 7.37 (2H, m), 7.28 (2H, m), 7.14 (1H, m), 7.01 (1H, d,  $J = 6.9$  Hz), 4.35 (3H, m), 3.30 (1H, dd,  $J = 13.7, 4.40$  Hz), 3.03 (1H, dd,  $J = 13.2, 8.5$  Hz); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>, 298 K)  $\delta$  178.3, 147.8, 139.9, 136.9, 135.2, 133.4, 132.2, 131.0, 130.7, 129.1, 128.2, 128.1, 125.7, 71.2, 40.4, 30.3; HRMS (FAB)  $m/z$  calc'd for C<sub>16</sub>H<sub>15</sub>NO<sub>5</sub>Se [M+H]: 382.0194, found 382.0191.

**Synthesis of aryl selenide-glutamate hydroxy-peptide (13).** Arylalkyl selenide  $\alpha$ -hydroxy acid **2** (0.091 g, 0.239 mmol, 1 eq), di-*tert*-butyl-L-glutamate (0.106 g, 0.359 mmol, 1.5 eq), and benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate (0.149 g, 0.287 mmol, 1.2 eq) were placed in a round-bottom flask under Ar (g) and dissolved in CH<sub>2</sub>Cl<sub>2</sub> (1.8 mL). To this was added *N*-methyilmorpholine (0.09 mL, 0.790 mmol, 3.3 eq) via syringe, and the reaction stirred for 24 hours. The reaction mixture was then diluted with EtOAc, and the resulting solution was washed with 1 M KHSO<sub>4</sub> (2 $\times$ ), H<sub>2</sub>O, 5% NaHCO<sub>3</sub> (2 $\times$ ), and brine. The organic layer was passed through a pad of Celite™, dried over MgSO<sub>4</sub>, and concentrated. The resulting solid was purified by flash column chromatography (30% EtOAc in hexanes) to afford hydroxy-peptide **13** as a yellow oil (0.134 g, 0.215 mmol, 90% yield).  $R_f$  = 0.49 (50% EtOAc in hexanes); <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>, 298 K)  $\delta$  7.98 (1H, d,  $J$  = 7.4 Hz), 7.45 (1H, d,  $J$  = 7.4 Hz), 7.36 (2H, m), 7.26 (2H, m), 7.10 (1H, m), 6.99 (1H, m), 4.46 (1H, m), 4.25 (1H, m), 3.30 (1H, dd,  $J$  = 14.0, 3.6 Hz), 2.96 (2H, m), 2.14 (3H, m), 1.86 (1H, m), 1.45 (18H, s); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>, 298 K)  $\delta$  172.7, 172.2, 171.0, 148.0, 140.9, 136.9, 135.0, 133.4, 132.2, 131.1, 130.9, 129.2, 128.3, 128.0, 125.7, 82.6, 80.9, 72.9, 52.1, 41.1, 31.6, 30.3, 28.3, 28.2, 27.9; HRMS (FAB)  $m/z$  calc'd for C<sub>29</sub>H<sub>38</sub>N<sub>2</sub>O<sub>8</sub>Se [M+H]: 623.1871, found 623.1881.

**Synthesis of tryptophan-aryl selenide-glutamate depsipeptide (14).** Hydroxy-peptide **13** (0.140 g, 0.225 mmol, 1 eq), *N* $\alpha$ -(*tert*-butoxycarbonyl)-*L*-tryptophan (0.137 g, 0.450 mmol, 2 eq), 4-(dimethylamino)pyridine (0.014 g, 0.113 mmol, 0.5 eq), and *N,N'*-dicyclohexylcarbodiimide (0.093g, 0.450 mmol, 2 eq) were placed in a round-bottom flask under Ar (g) and dissolved in CH<sub>2</sub>Cl<sub>2</sub> (5 mL). After stirring for 48 hours, the

reaction mixture was diluted with EtOAc. The solution was washed with 1 M KHSO<sub>4</sub> (2×), H<sub>2</sub>O, 5% NaHCO<sub>3</sub> (2×), and brine. The organic layer was filtered through a pad of Celite™, dried over MgSO<sub>4</sub>, and concentrated. The resulting solid was purified by flash column chromatography (35% EtOAc in hexanes) to afford depsipeptide **14** as a pale yellow, non-crystalline solid (0.0817 g, 0.0900 mmol, 40% yield).  $R_f = 0.34$  (50% EtOAc in hexanes); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>, 298 K)  $\delta$  8.52 (1H, b), 7.98 (1H, m), 7.57 (1H, d,  $J = 7.9$  Hz), 7.41 (1H, d,  $J = 7.7$  Hz), 7.34 (3H, m), 7.18 (3H, m), 7.10 (2H, m), 7.02 (1H, s), 6.91 (1H, m), 5.25 (1H, m), 4.98 (1H, d,  $J = 7.5$  Hz), 4.57 (1H, dd,  $J = 12.6, 5.3$  Hz), 4.41 (1H, m), 4.24 (2H, s), 3.17 (2H, m), 2.20 (1H, m), 2.08 (2H, m), 1.79 (1H, m), 1.48 (9H, s), 1.45 (9H, s), 1.31 (9H, s). <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>, 298 K)  $\delta$  172.4, 171.2, 169.0, 156.0, 148.0, 139.7, 136.7, 136.4, 135.1, 133.3, 132.1, 131.3, 131.0, 129.0, 128.2, 128.1, 128.0, 125.7, 123.9, 122.3, 122.3, 119.8, 118.9, 111.5, 109.6, 82.3, 80.9, 80.1, 75.1, 54.6, 52.4, 37.7, 34.2, 31.8, 30.3, 28.5, 28.4, 28.2, 27.6; HRMS (ESI)  $m/z$  calc'd for C<sub>45</sub>H<sub>56</sub>N<sub>4</sub>O<sub>11</sub>Se [M+H]: 909.3229, found 909.3238.

**Synthesis of selenacyclopentane (15).** Depsipeptide **12** (0.0722g, 0.088 mmol, 1 eq) was placed in a pyrex reaction vessel and dissolved in acetonitrile (125 mL). To this was added dithiothreitol (1.36 g, 8.8 mmol, 100 eq) and 20 mM phosphate buffer, pH 7.6 (125 mL). The resulting solution was stirred under N<sub>2</sub> (g), and a 450 W medium-pressure mercury-vapor UV immersion lamp (ACE Glass), filtered with a pyrex glass absorption sleeve and equipped with a water cooling jacket, was assembled and attached to the reaction vessel. The progress of the reaction was followed by ESI-MS. After 1 hour of photolysis (at which point the temperature of the reaction had increased from 25 °C to 32 °C) the two major  $m/z$  ratios seen in the mass spectrum of the reaction were the

depsipeptide **12** ( $[M+Na^+] = 844$   $m/z$ ) and the nitrobenzyl-deprotected selenol ( $[M+Na^+] = 709$   $m/z$ ). After 5 hours of photolysis, the  $m/z$  ratio attributed to **12** ( $[M+Na^+] = 844$   $m/z$ ) had diminished to a negligible level while the  $m/z$  ratios corresponding to the nitrobenzyl-deprotected selenol ( $[M+Na^+] = 709$   $m/z$ ), the nitrobenzyl-deprotected diselenide ( $[M+Na^+] = 1391$   $m/z$ ), the olefin-containing depsipeptide derived from the oxidative elimination of the selenium ( $[M+Na^+] = 627$   $m/z$ ), and the desired selenacyclopentane **15** ( $[M+Na^+] = 444$   $m/z$ ) persisted. At this time, the reaction was removed from the photoreactor, heated to 70 °C, and monitored by ESI-MS. After 3 hours of heating at this temperature, both nitrobenzyl-deprotection products ( $m/z$  ratios 709 and 1391) were no longer detectable, but the  $m/z$  ratio attributed to the desired selenacyclopentane **15** ( $[M+Na^+] = 444$   $m/z$ ) and a  $m/z$  ratio corresponding to its dimer ( $[M+Na^+] = 865$   $m/z$ ) remained. The resulting mixture was extracted with EtOAc (2×), dried over MgSO<sub>4</sub>, and concentrated. The crude product was purified twice by flash column chromatography (both began with 11% EtOAc in hexanes then were changed to 33% EtOAc in hexanes after the DTT eluted, both were dry loaded in CH<sub>2</sub>Cl<sub>2</sub>) to afford selenacyclopentane **15** as a yellow oil (0.0107 g, 0.0255 mmol, 29% yield).  $R_f = 0.28$  (33% EtOAc in hexanes);  $[\alpha]_D^{24} = -16.0^\circ$  ( $c = 1$ , CHCl<sub>3</sub>); <sup>1</sup>H NMR (500 MHz, CDCl<sub>3</sub>, 298 K)  $\delta$  7.08 (1H, d,  $J = 13.5$  Hz), 4.44 (1H, dt,  $J = 13.5, 8$  Hz), 4.08 (1H, m), 3.15 (1H, m), 2.97 (1H, m), 2.37 (1.5H, m), 2.26 (1.5H, m), 2.14 (4H, m), 1.92 (1H, m), 1.47 (9H, s), 1.44 (9H, s); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>, 298 K)  $\delta$  172.3, 172.1, 170.8, 82.3, 80.7, 52.845.4, 37.4, 32.4, 31.5, 28.1, 28.0, 27.5, 27.1. HRMS (TOF)  $m/z$  calc'd for C<sub>18</sub>H<sub>31</sub>NO<sub>5</sub>Se  $[M+H]^+$ : 422.1446, found 422.1465.

**Synthesis of aryl selenacyclopentane (16).** Depsipeptide **14** (0.0230 g, 0.0250 mmol, 1 eq) was placed in a pyrex reaction vessel and dissolved in acetonitrile (125 mL). To this was added dithiothreitol (0.0390 g, 0.250 mmol, 10 eq) and pH 8 H<sub>2</sub>O (125 mL). The resulting solution was stirred under N<sub>2</sub> (g), and a 450 W medium-pressure mercury-vapor UV immersion lamp (ACE Glass), filtered with a uranium glass absorption sleeve and equipped with a water cooling jacket, was assembled and attached to the reaction vessel. The progress of the reaction was followed by ESI-MS. After 1 hour of photolysis, three *m/z* ratios were seen in the mass spectrum of the reaction: the depsipeptide **14** ([M+Na<sup>+</sup>] = 931 *m/z*), the nitrobenzyl deprotected aryl selenol ([M+Na<sup>+</sup>] = 796 *m/z*), and the desired aryl selenacyclopentane **16** ([M+Na<sup>+</sup>] = 492 *m/z*). No *m/z* ratio corresponding to the nitrobenzyl deprotected diselenide ([M+Na<sup>+</sup>] = 1567 *m/z*) was observed. After 2 hours, both *m/z* ratios attributed to **14** ([M+Na<sup>+</sup>] = 931 *m/z*) and the nitrobenzyl-deprotected aryl selenol ([M+Na<sup>+</sup>] = 796 *m/z*) had diminished to a negligible level while a *m/z* ratio corresponding to the desired aryl selenacyclopentane **16** ([M+Na<sup>+</sup>] = 492 *m/z*) persisted. At this time, the reaction was removed from the photoreactor. The acetonitrile was then removed *in vacuo*, and the resulting aqueous solution was extracted with Et<sub>2</sub>O (3×), washed with brine, dried over MgSO<sub>4</sub>, and concentrated. The resulting yellow oil was purified by flash column chromatography (15% EtOAc in hexanes) to afford aryl selenacyclopentane **16** as a pale yellow oil (0.00843 g, 0.0180 mmol, 72% yield). *R<sub>f</sub>* = 0.59 (30% EtOAc in hexanes); [α]<sup>24</sup><sub>D</sub> = -80.5° (c = 1, CHCl<sub>3</sub>); <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>, 298 K) δ 7.30 (1H, m), 7.20 (1H, m), 7.12 (2H, m), 7.29 (2H, m), 6.92 (1H, d, *J* = 7.41 Hz), 4.50 (1H, dd, *J* = 8.2, 5.2 Hz), 4.43 (1H, dd, *J* = 7.8, 4.7 Hz), 2.25 (2H, m), 2.15 (1H, m), 1.91 (1H, m), 1.44 (9H, s), 1.41 (9H, s); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>, 298

K)  $\delta$  172.4, 171.4, 170.7, 141.7, 135.3, 128.0, 125.9, 125.7, 125.5, 82.6, 81.0, 53.1, 45.9, 41.7, 31.6, 29.9, 28.3, 28.1; HRMS (ESI)  $m/z$  calc'd for  $C_{22}H_{31}NO_5Se$  [M+H]: 470.1446, found 470.1469.

**Synthesis of the cyanomethyl ester of 2.** Compound **2** (0.12 g, 0.032 mmol, 1 eq) was added to a 20 mL scintillation vial. To this was added chloroacetonitrile (0.10 mL, 1.6 mmol, 50 eq) and triethylamine (0.013 mL, 0.095 mmol, 3 eq). The resulting mixture stirred for 6 hours. Then 10 mL of  $H_2O$  was added and the organics were extracted with  $CH_2Cl_2$  (3 $\times$ ), washed with brine, dried over  $Na_2SO_4$ , concentrated, and then purified by flash column chromatography (50% EtOAc in hexanes) to afford the cyanomethyl ester of **2** as a yellow oil (0.010 g, 0.024 mmol, 76% yield).  $R_f$  = 0.40 (50% EtOAc in hexanes);  $^1H$  NMR (300 MHz,  $CDCl_3$ , 298 K)  $\delta$  8.01 (1H, d,  $J$  = 9.5 Hz), 7.52 (1H, d,  $J$  = 7.6 Hz), 7.34 (3H, m), 7.22 (1H, m), 7.17 (1H, m), 6.97 (1H, m), 4.77 (2H, b), 4.34 (3H, b), 3.16 (1H, dd,  $J$  = 13.7, 4.5 Hz), 2.94 (1H, dd,  $J$  = 13.8, 8.5 Hz), 2.64 (1H, d,  $J$  = 5.6 Hz);  $^{13}C$  NMR (75 MHz,  $CDCl_3$ , 298 K)  $\delta$  172.7, 139.6, 137.2, 135.1, 133.3, 132.0, 132.0, 130.9, 130.6, 129.2, 128.3, 128.3, 125.7, 114.0, 71.1, 49.1, 40.6, 30.5; HRMS (FAB+)  $m/z$  calc'd for  $C_{18}H_{17}N_2O_5Se$  [M+H]: 421.0303, found 421.0303. A similar protocol was used to synthesize the cyanomethyl ester of **1** and is described in the theses of Dr. Amy L. Eastwood<sup>23</sup> and Dr. Niki M. Zacharias.<sup>24</sup>

**Synthesis of 4PO-protected nitrophenylalanine 18.** *L*-2-nitrophenylalanine (CSPS Pharmaceuticals) (1.5 g, 7.1 mmol, 1 eq) was added to a 20 mL round-bottom flask under Ar (g) and suspended in 20 mL of THF and 10 mL of  $H_2O$ . The resulting suspension was placed in an ice bath. To this was added triethylamine (2.1 mL, 15 mmol), 2.1 eq). Pent-4-enoic anhydride (1.5 mL, 8.5 mmol, 1.2 eq) was then added dropwise. After 1 hour of

stirring, an addition 0.5 mL of triethylamine (3.6 mmol) and 0.5 mL of pent-4-enoic anhydride (2.8 mmol) was added. The solution was stirred for 1 more hour and then 50 mL of 0.2 M NaHSO<sub>4</sub> was added. The pH of the resulting solution was increased to pH 9 by addition of 2M NaOH. The organics were extracted with Et<sub>2</sub>O and discarded. The pH of the aqueous layer was then decreased to pH 2 via addition of 6 M HCl. The organics were extracted with EtOAc (3×), washed with 0.2 M NaHSO<sub>4</sub>, washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, filtered, and concentrated. The resulting yellow liquid was purified by flash column chromatography (0.9% formic acid and 32% hexanes in EtOAc) to afford 4PO-protected nitrophenylalanine **18** as a yellow oil (1.8 g, 6.2 mmol, 87% yield).  $R_f = 0.46$  (0.9% formic acid and 32 % hexanes in EtOAc); <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>, 298 K) δ 9.92 (1H, b), 7.95 (1H, d,  $J = 8.2$  Hz), 7.59 (1H, m), 7.46 (2H, m), 5.69 (1H, m), 4.98 (3H, m), 3.62 (1H, dd,  $J = 20, 6.8$  Hz), 3.34 (1H, dd,  $J = 24, 9.4$  Hz) 2.19 (2H, m); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>, 298 K) δ 174.1, 173.7, 149.7, 136.4, 133.3, 132.8, 131.5, 128.3, 124.9, 115.8, 53.0, 35.3, 34.3, 29.2.

**Synthesis of 4PO- *t*-butyl-protected nitrophenylalanine 19.** Compound **18** (0.33 g, 1.1 mmol, 1 eq) was placed in a 2-neck round-bottom flask under Ar (g) and dissolved in THF (5 mL) and cyclohexane (5 mL). A solution of *tert*-butyl-2,2,2-trichloroacetimidate (0.82 mL, 4.6 mmol, 4 eq) in cyclohexane (5 mL) was added simultaneously with boron trifluoride diethyletherate (0.049 mL, 0.40 mmol, 0.35 eq). The reaction stirred for 1 hour until it was quenched with 20 mL of saturated NaHCO<sub>3</sub> (aq). The organics were extracted with Et<sub>2</sub>O (3×), washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated. The resulting white sludge was suspended in hexanes, filtered, and then purified by flash column chromatography (30% EtOAc in hexanes) to afford 4PO- *t*-butyl-protected

nitrophenylalanine **19** as a yellow oil (0.32 g, 0.90 mmol, 80% yield).  $R_f = 0.30$  (30% EtOAc in hexanes);  $^1\text{H NMR}$  (300 MHz,  $\text{CDCl}_3$ , 298 K)  $\delta$  7.89 (1H, d,  $J = 8.4$  Hz), 7.43 (3H, m), 6.33 (1H, d,  $J = 8.2$ ), 5.65 (1H, m), 4.89 (3H, m), 3.46 (1H, dd,  $J = 13.8, 6.1$  Hz), 3.22 (1H, dd,  $J = 13.8, 8.8$  Hz), 2.20 (2H, m), 1.37 (9H, m);  $^{13}\text{C NMR}$  (75 MHz,  $\text{CDCl}_3$ , 298 K)  $\delta$  172.0, 170.5, 149.7, 136.8, 133.0, 132.8, 132.0, 128.0, 124.8, 115.5, 82.6, 53.1, 35.4, 35.1, 29.2, 27.8; HRMS (FAB+)  $m/z$  calc'd for  $\text{C}_{18}\text{H}_{25}\text{N}_2\text{O}_5$  [M+H]: 349.1763, found 349.1753.

**Synthesis of 4PO- *t*-butyl- NVOC-protected nitrophenylalanine 20.** Compound **19** (1.3 g, 3.5 mmol, 1 eq) was placed in a 2-neck round-bottom flask under Ar (g) and dissolved in THF (50 mL) and MeOH (25 mL) at 0 °C. To this was added 10 wt % palladium on activated carbon (0.060 g) and sodium borohydride (0.26 g, 7.0 mmol, 2 eq). The reaction was followed by TLC using ninhydrin staining. The solution was stirred for 40 minutes and was then quenched with water and filtered through a pad of Celite™. The filtrate was extracted with  $\text{Et}_2\text{O}$  (3×), washed with brine, dried over  $\text{Na}_2\text{SO}_4$ , and concentrated to afford a pale yellow oil. The resulting liquid was purified by flash column chromatography (50% EtOAc in hexanes) to afford 4PO- *t*-butyl- NVOC-protected nitrophenylalanine **20** as a pale yellow oil (0.93 g, 2.9 mmol, 84% yield).  $R_f = 0.40$  (50% EtOAc in hexanes);  $^1\text{H NMR}$  (300 MHz,  $\text{CDCl}_3$ , 298 K)  $\delta$  7.01 (1H, t,  $J = 7.6$  Hz), 6.89 (1H, d,  $J = 7.3$  Hz), 6.62 (2H, m), 5.76 (1H, m), 5.00 (2H, m), 4.60 (1H, m), 3.05 (1H, dd,  $J = 14.1, 4.1$  Hz), 2.76 (1H, dd,  $J = 14.4, 9.0$  Hz), 2.35 (2H, m), 1.34 (9H, s);  $^{13}\text{C NMR}$  (75 MHz,  $\text{CDCl}_3$ , 298 K)  $\delta$  172.4, 171.3, 145.3, 136.8, 131.4, 128.1, 120.7, 117.7, 115.6, 82.4, 52.4, 35.7, 29.4, 27.8. HRMS (FAB+)  $m/z$  calc'd for  $\text{C}_{18}\text{H}_{25}\text{N}_2\text{O}_5$  [M+]: 318.1944, found 318.1931.

**Synthesis of 4PO- NVOC-protected nitrophenylalanine 21.** Compound **20** (0.20 g, 0.63 mmol, 1 eq) was placed in a 20 mL scintillation vial and suspended in 10 mL of CH<sub>2</sub>Cl<sub>2</sub>. To this was added K<sub>2</sub>CO<sub>3</sub> (0.18 g, 1.3 mmol, 2 eq) and 6-nitroveratryloxycarbonyl (NVOC) chloride (0.17 g, 0.63 mmol, 1 eq). The reaction stirred for 1 hour and then 20 mL of H<sub>2</sub>O was added. The organics were extracted with CH<sub>2</sub>Cl<sub>2</sub> (3×), washed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, concentrated, and then purified by flash column chromatography (40% EtOAc in hexanes) to afford 4PO- NVOC-protected nitrophenylalanine **21** as a yellow oil (0.24 g, 0.43 mmol, 68% yield). *R<sub>f</sub>* = 0.30 (40% EtOAc in hexanes); <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>, 298 K) δ 7.69 (1H, s), 7.33 (1H, b), 7.25 (1H, m), 7.06 (3H, m), 6.63 (1H, m), 5.68 (3H, m), 4.99 (2H, m), 4.40 (1H, m), 3.96 (3H, s), 3.90 (3H, s), 3.17 (1H, dd, *J* = 14.4, 3.6 Hz), 2.83 (1H, dd, *J* = 14.1, 9.6 Hz), 2.27 (4H, m), 1.32 (9H, s); <sup>13</sup>C NMR (75 MHz, CDCl<sub>3</sub>, 298 K) δ 172.9, 170.6, 153.8, 153.8, 147.8, 139.2, 136.7, 136.4, 131.4, 128.6, 128.0, 126.2, 123.4, 121.5, 116.0, 109.9, 108.0, 83.1, 63.7, 56.6, 56.3, 53.2, 35.5, 35.4, 29.5, 27.8.

**Synthesis of 4PO- NVOC-protected aniline 22.** Compound **21** (0.10 g, 0.18 mmol) was placed in a 2-neck round-bottom flask under Ar (g) and dissolved in CH<sub>2</sub>Cl<sub>2</sub> (3 mL). To this was added trifluoroacetic acid (3 mL) and the solution stirred for 16 hours. The reaction mixture was concentrated to afford an orange liquid, which was triturated with ether. The resulting yellow solid was dissolved in EtOAc (3×), rinsed with 1 N HCl, rinsed with H<sub>2</sub>O, and then added to 5% NaHCO<sub>3</sub>. The organic layer (colorless) was discarded, and the pH of the aqueous layer was decreased to pH 2 by the addition of 6 N HCl. The organics were extracted with EtOAc (3×), rinsed with brine, dried over Na<sub>2</sub>SO<sub>4</sub>, and concentrated, and then purified by flash column chromatography (1%

formic acid and 15% hexanes in EtOAc) to afford 4PO, NVOC-protected aniline **22** as a yellow oil (0.80 g, 0.16 mmol, 89% yield).  $R_f = 0.38$  (1% formic acid and 15% hexanes in EtOAc);  $^1\text{H NMR}$  (300 MHz, MeOD, 298 K)  $\delta$  9.01 (1H, b), 7.75 (1H, s), 7.40 (1H, m), 7.21 (4H, m), 5.68 (1H, m), 5.56 (2H, s), 4.91 (2H, m), 4.70 (1H, m), 3.91 (6H, s), 3.29 (1H, m), 2.97 (1H, dd  $J = 14.5, 9.4$  Hz), 2.18 (4H, m).

**Synthesis of the cyanomethyl ester of 22.** Compound **22** (0.048 g, 0.096 mmol, 1 eq) was added to a 20 mL scintillation vial. To this was added chloroacetonitrile (0.30 mL, 4.8 mmol, 50 eq) and triethylamine (0.040 mL, 0.29 mmol, 3 eq). The resulting mixture stirred for 6 hours. Then 10 mL of  $\text{H}_2\text{O}$  was added and the organics were extracted with  $\text{CH}_2\text{Cl}_2$  (3 $\times$ ), washed with brine, dried over  $\text{Na}_2\text{SO}_4$ , concentrated, and then purified by flash column chromatography (50% EtOAc in hexanes) to afford the cyanomethyl ester of **22** as a yellow oil (0.041 g, 0.076 mmol, 79% yield).  $R_f = 0.40$  (50% EtOAc in hexanes);  $^1\text{H NMR}$  (300 MHz,  $\text{CDCl}_3$ , 298 K)  $\delta$  7.72 (2H, m), 7.24 (4H, m), 6.68 (1H, b), 5.66 (2H, m), 4.95 (2H, m), 4.47 (3H, m), 3.96 (6H, s), 3.19 (1H, dd  $J = 14.3, 6.4$  Hz), 3.06 (1H, dd,  $J = 14.5, 8.2$ ), 2.28 (4H, m).

**Synthesis of the cyanomethyl ester of 7.** Compound **7** (0.40 g, 1.9 mmol, 1 eq) was added to a 20 mL scintillation vial. To this was added chloroacetonitrile (3.0 mL, 4.8 mmol, 25 eq) and triethylamine (0.80 mL, 5.7 mmol, 3 eq). The resulting mixture stirred for 6 hours. Then 20 mL of  $\text{H}_2\text{O}$  was added and the organics were extracted with  $\text{CH}_2\text{Cl}_2$  (3 $\times$ ), washed with brine, dried over  $\text{Na}_2\text{SO}_4$ , concentrated, and then purified by flash column chromatography (40% EtOAc in hexanes) to afford the cyanomethyl ester of **7** as a yellow oil (0.33 g, 1.3 mmol, 70% yield).  $R_f = 0.39$  (50% EtOAc in hexanes);  $^1\text{H NMR}$  (300 MHz,  $\text{CDCl}_3$ , 298 K)  $\delta$  7.95 (1H, d,  $J = 7.4$  Hz), 7.61 (1H, dd,  $J = 15.1, 7.2$ ), 7.48

(2H, m), 4.83 (2H, s), 4.64 (1H, m), 3.55 (1H, dd,  $J = 13.9, 4.6$  Hz), 3.25 (1H, dd,  $J = 14.1, 8.2$ ), 2.96 (1H, b);  $^{13}\text{C}$  NMR (75 MHz,  $\text{CDCl}_3$ , 298 K)  $\delta$  172.4, 149.6, 133.4, 133.3, 130.9, 128.4, 125.0, 113.9, 70.4, 49.2, 37.1; HRMS (FAB+)  $m/z$  calc'd for  $\text{C}_{11}\text{H}_{11}\text{N}_2\text{O}_5$  [M+]: 251.0668, found 251.0677.

**Nonsense Suppression *in vitro*.** Rabbit Reticulocyte Lysate (Promega) and Wheat Germ Lysate (Rabbit Reticulocyte Lysate/Wheat Germ Extract Combination System from Promega) translation systems were used for *in vitro* transcription according to the manufacturer's protocol. All materials (*e.g.*, 35  $\mu\text{L}$  Rabbit Reticulocyte lysate, 1.5  $\mu\text{L}$  amino acid mix, 1  $\mu\text{L}$  RNase inhibitor, 2.5  $\mu\text{L}$  water, 6  $\mu\text{L}$  mRNA at 1mg/mL and either 1  $\mu\text{L}$  tRNA at 1 mg/mL or 1  $\mu\text{L}$  water) were mixed and incubated at 30 °C for 3 hours. The resulting samples were stored at  $-80$  °C until needed.

Aliquots of the *in vitro* translation reactions (5 mL) were used for photolysis and base hydrolysis experiments. For photolysis experiments, the aliquots were irradiated for 10 minutes to 1 hour using a 1000 W Hg/Xe arc lamp and UG-11 and WG-335 filters or a 288 W Hg lamp equipped with a 360 nm band-pass filter (BLAK-RAY Longwave Ultraviolet Lamp, Ultraviolet Products, Inc.) at a distance of 15-30 cm. For some experiments, the sample was heated (to various temperatures up to 90 °C) after irradiation. For base hydrolysis experiments, aliquots were treated with concentrated  $\text{NH}_4\text{OH}$  as described in England *et al.*<sup>10</sup>

Samples for SDS-polyacrylamide gel electrophoresis (PAGE) were prepared by mixing 5  $\mu\text{L}$  of the *in vitro* translation mix with 5  $\mu\text{L}$  of  $2 \times$  SDS loading buffer (100 mM tris chloride at pH 6.8, 4% SDS, 0.2% bromophenol blue, 20% glycerol). Samples (10  $\mu\text{L}$  each) were loaded onto pre-poured 7.5 or 12% tris chloride gels for SDS-PAGE. For

Western blotting, protein was transferred from the gel to nitrocellulose, which was then blotted with a mouse anti-hemagglutinin (HA) primary antibody that was in turn treated with a goat anti-mouse secondary antibody conjugated to horseradish-peroxidase for detection by chemiluminescence.

**Nonsense Suppression in *Xenopus* oocytes.** Nonsense suppression was performed using techniques described previously<sup>3, 33</sup> on Shaker B cDNA in the pAMV vector. An appropriate codon (as described in the text) was introduced at the site of interest by the standard Stratagene QuickChange protocol and verified through sequencing. cDNA was linearized with the restriction enzyme NotI and mRNA was prepared by *in vitro* transcription using the mMessage Machine T7 kit (Ambion).

Stage V-VI *Xenopus laevis* oocytes were injected with 1 ng of mRNA per oocyte in a single 75 nL injection. For nonsense suppression experiments, each cell was injected with 75 nL of a 1:1 mixture of mRNA (20-25 ng of total mRNA) and tRNA (10-25 ng). Unnatural amino acids bearing 4PO protecting groups were deprotected prior to injection by incubating the sample with saturated I<sub>2</sub> (aq), which was prepared by making a saturated solution in water that was sonicated for 5 minutes and then heated at 60 °C for another 15 minutes. Oocytes were incubated at 18 °C for or 24-48 hours after injection. Wild-type recovery control experiments (injection of tRNA appended to the natural amino acid) were performed to evaluate the fidelity of the nonsense suppression experiments. Additional controls, including injections of mRNA only and mRNA with 76-mer THG73 (the misacylation control), were also performed.

Two-electrode voltage clamp electrophysiology was used to measure the functional effects of each mutation. Electrophysiology recordings were performed after

injection and incubation as described above using the OpusXpress 6000A instrument (Axon Instruments) at a holding potential of  $-80$  mV. The running buffer was a  $\text{Ca}^{2+}$  free ND96 solution (96 mM NaCl, 2 mM KCl, 1 mM  $\text{MgCl}_2$ , and 5 mM HEPES, pH 7.5). Currents were measured during depolarizing jumps from the holding potential to  $+70$  mV in 25 mV increments.

For photolysis experiments, oocytes were irradiated for 10 minutes to 4 hours (with chilling) using a 1000 W Hg/Xe arc lamp using UG-11 and WG-335 filters or a 288 W Hg lamp equipped with a 360 nm band-pass filter (BLAK-RAY Longwave Ultraviolet Lamp, Ultraviolet Products, Inc.) at a distance of 15-30 cm.

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