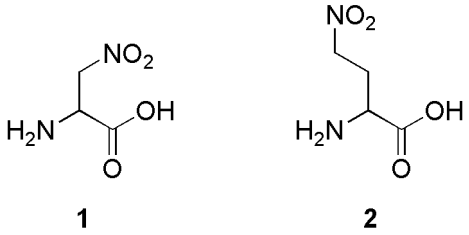
Appendix A: The Synthesis of Nitro Amino Acids: Nitroalanine and Nitrohomoalanine

A.1 Introduction

For our study of the conserved Asp, D89, in the nicotinic acetylcholine receptor (nAChR), we wished to study the role of the residue's negative charge in receptor function[1]. Also, we had hoped to study the conserved binding site residue, D3.32, in the aminergic class of G-protein-coupled receptors (GPCRs). This Asp has been suggested to form an electrostatic interaction with positively charged monoamine ligands[2–5]. We therefore decided to synthesize unnatural amino acids that could subtly probe the contribution of the negative charge of Asp or Glu to receptor structure and function.

The nitro group provides a neutral analog to the carboxylate group that is isosteric and isoelectronic (Figure 2.5). As described in Chapter 2, D89N mutations not only neutralize side-chain charge, but also introduce an electrostatic clash through the amide moiety. Another researcher in our group has previously synthesized the keto analog to Asp, 2-amino-4-ketopentanoic acid (Akp)[6], and we have shown in Chapter 2 that this amino acid relieves the electrostatic clash caused by Asn. But Akp destroys the symmetry of Asp, which may disrupt the hydrogen bonding network of the native residue. A nitro analog of Asp would neutralize the negative charge while maintaining the side chain's symmetry and avoiding steric clashes. A significant difference between

the nitro group and the carboxylate is the substantially weaker hydrogen-bond-accepting ability of the nitro group (energetic differences are between 1.5 and 2.0 kcal/mol)[7].



1 **2**

Figure A.1. Structures of nitroalanine (**1**) and nitrohomoalanine (**2**)

This appendix describes the synthesis of nitroalanine (Noa) and nitrohomoalanine (Nha; Figure A.1). While Nha could be synthesized and adapted to our nonsense suppression methodology, Noa was incompatible with a crucial transformation.

A.2 Results and Discussion

A.2.1 Noa Synthesis

To synthesize Noa, we used a literature procedure (Figure A.2, ***i*** through ***iii***) that began with a fully protected glycine (**3**)[8,9]. Boc-2-bromoglycine *tert*-butyl ester (**4**) was synthesized from **3** through photobromination. After reaction of **4** with methyl nitronate and acid deprotection, we were able to produce Noa (**1**). The standard NVOC protection of the free amine was performed without issue.

But when we attempted to synthesize the NVOC-nitroalanine cyanomethyl ester (**8b)**, we mainly produced NVOC-dehydroalanine cyanomethyl ester (**8a**). This side product was the result of deprotonation at the α carbon and elimination of the nitro group to yield the α, β unsaturated amino acid. Some of the desired cyanomethyl ester was

produced, but we were unable to separate it from **8a** through flash chromatography; we believed that the compound degraded while on the column. Attempts at running the cyanomethyl reaction with weaker bases, such as Na_2CO_3[10], also produced dehydroalanine.

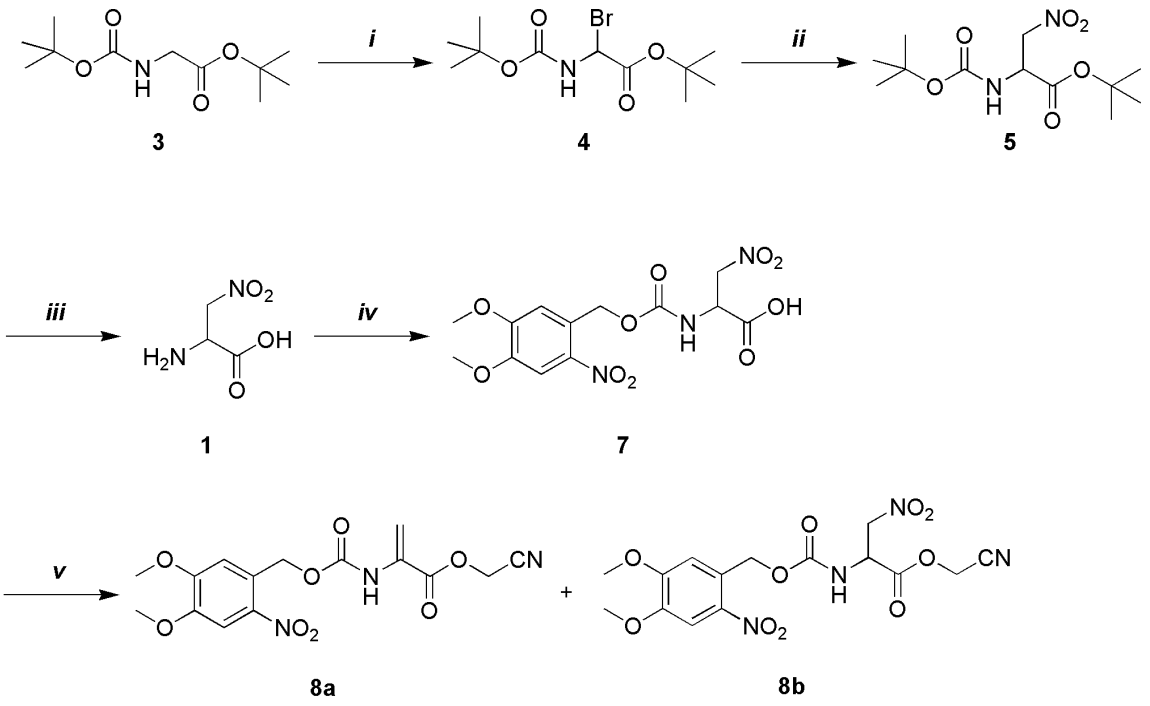


Figure A.2. Synthetic route for the attempted synthesis of NVOC-nitroalanine cyanomethyl ester. ***i***: *N*-bromosuccinimide, *hv*. ***ii***: *n*-BuLi, CH_3NO_2, THF, HMPA. ***Iii***: 1:1 TFA / CH_2Cl_2. ***iv***: NVOC-Cl, 2 eq. Na_2CO_3, 1:1 H_2O/dioxanes. ***v***: 2 eq. Et_3N, $ClCH_2CN$; or 2 eq. Na_2CO_3, 2 eq. $ClCH_2CN$, DMSO

The elimination of the nitro group in Noa may be unavoidable when transforming the free amino acid into a form that is compatible with dCA coupling. β-nitro amino acids have been synthesized as a facile route to dehydro amino acids in synthetic peptides[8,9]. Because dehydro amino acids are not stable enough for solid-phase peptide synthesis, β-nitro amino acids are incorporated into the peptide and subsequent treatment of the full peptide with base eliminates the nitro group, yielding the dehydro amino acid.

Basic conditions during the cyanomethyl ester formation step and the dCA coupling procedure may prevent us from incorporating Noa through standard means.

One alternative route could involve oxidation of the primary amine of 2,3-diaminopropionic acid (Dap) to a nitro group. Early in our attempts to synthesize Noa, we investigated the zirconium alkoxide catalyzed oxidation of primary amines[11–13]. This transformation could be performed on the cyanomethyl ester of Dap to produce the cyanomethyl ester of Noa. Of course, if the nitro group is eliminated by dCA coupling conditions, this route will also not be viable. Any attempt to revisit the Noa synthesis should begin with submitting **5** to dCA coupling conditions to determine how stable the NO_2 group is during coupling.

A.2.2 Nha Synthesis

Unlike Noa, Nha was found to be adaptable to both cyanomethyl ester formation and dCA coupling procedures. The synthesis of Nha (Figure A.3) began with the reaction of a nucleophilic protected glycine (**10**) with nitroethylene (**9**) following a literature procedure[14,15]. Nitroethylene was prepared from 2-nitroethanol following a literature preparation[16]. Deprotection of the protected Nha (**11**) by acid yielded the free amino acid (**2**). Standard NVOC protection and cyanomethyl ester formation procedures were used to produce **14**. dCA-Nha was formed using our standard method.

As an aside, Nha could also be synthesized from dehydroalanine. Reaction of methyl nitronate with protected dehydroalanine has been shown to yield Nha[17]. Because

the distillation of nitroethylene is cumbersome (see A.3 for details), this route may serve as an easier means to synthesize Nha.

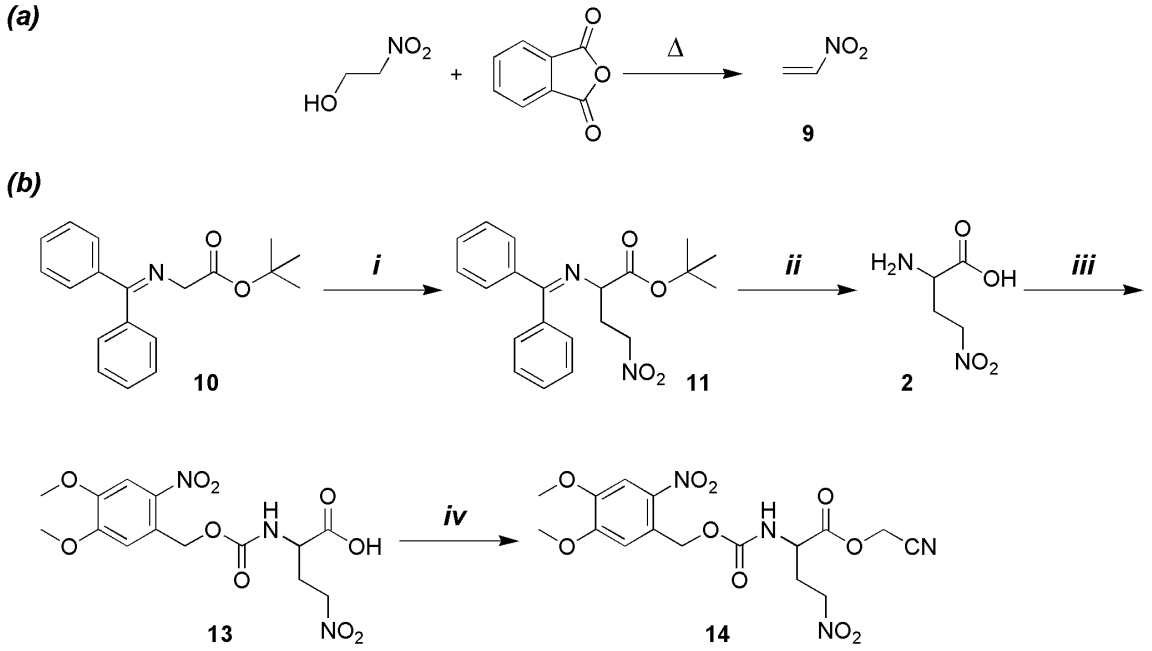


Figure A.3. Synthesis of NVOC-nitrohomoalanine cyanomethyl ester. ***(a)*** Preparation of nitroethylene. ***(b)*** Synthetic route for the synthesis of nitrohomoalanine. ***i***: 1) 1 eq. LDA, THF; 2) nitroethylene, THF. ***ii*****:** 1 N HCl. ***iii***: NVOC-Cl, 2 eq. Na_2CO_3, 1:1 H_2O/dioxanes. ***iv***: 2 eq. Et_3N, $ClCH_2CN$

A.3 Materials and Methods

*Synthesis of N-Boc-2-Bromoglycine t-Butyl ester (**4**)*

A solution of *N*-Boc-glycine *tert*-Butyl ester (**3**; 463 mg, 2 mmol) and *N*-bromosuccinimide (356 mg, 2 mmol) in 23 mL of dry CCl_4 was stirred for 1 h in front of a 1 kW xenon lamp without the WG-335 and UG-11 filters (without the filters, there was greater than 75% transmission for wavelengths greater than 250 nm). The solution was then filtered and evaporated to yellow crystals that were stored under Ar at 4ºC. ^{1}H-NMR ($CDCl_3$) δ 1.52 (s, 9H), 5.92 (d, 1H), 6.24 (d, 1H).

*Synthesis of N-Boc-Nitroalanine tert-Butyl ester (**5**)*

Nitromethane (216 μL, 4mmol) was stirred with 20 mL THF and 4 mL HMPA at -78ºC under Ar. *n*-BuLi (1.6 mL of a 2.5 M solution in hexanes, 4 mmol) was added dropwise to the nitromethane solution to produce the methyl nitronate. A solution of **4** (618 mg, 2 mmol) in 2 mL THF was added dropwise to the reaction mixture and allowed to stir for 4 h at -78ºC. The reaction was then quenched with 3 mL of acetic acid and the reaction flask was allowed to warm to room temperature. After dilution of the reaction mixture with 25 mL of ethyl acetate, three washes with brine, and drying over Na_2SO_4, the solution was evaporated to a solid. Crude product was purified on a flash silica column using a 5:1 hexanes / ethyl acetate solvent system to yield 330 mg of pure product (57% yield). ^{1}H-NMR ($CDCl_3$) δ 1.46 (s, 9H), 1.50 (s, 9H), 4.6 (m, 1H), 4.78 (m, 1H), 4.92 (m, 1H), 5.47 (d, 1H).

*Synthesis of Nitroalanine (**1**)*

N-Boc-nitroalanine *tert*-butyl ester (330 mg, 1.14 mmol) was dissolved in a 1:1 solution of TFA and dichloromethane (15 mL each). After 1 h, the solution was evaporated to dryness to yield 245 mg of **1**. Product was taken onto the next step without characterization.

*Synthesis of NVOC-Nitroalanine (**7**)*

Nitroalanine (245 mg, 0.99 mmol) and Na_2CO_3 (212 mg, 2 mmol) were dissolved in water (20 mL). To this solution, NVOC-Cl (300 mg, 1.09 mmol) in dioxane (20 mL) was added and stirred for 4 h at room temperature. The reaction was evaporated to half of the reaction volume and then diluted with 40 mL of water. The solution was extracted with ether (40 mL) until the organic layer was no longer colored. The aqueous layer was acidified with HCl to a pH of ~ 2 (solution became cloudy) and extracted with dichloromethane until organic layer was clear. The organic layers were dried and evaporated to yield 194 mg of **7** (52%). ^{1}H-NMR ($CDCl_3$) δ 3.97 (s, 1H), 4.01 (s, 1H), 4.88 (m, 1H), 4.94 (m, 1H), 5.07 (m, 1H), 5.59 (s, 2H), 5.90 (d, 1H), 6.98 (s, 1H), 7.74 (s, 1H). ES-MS calculated for $C_{13}H_{15}N_3O_{10}$: 373.08, found *m/z* $(M+Na)^+$: 395.8. Crude product was taken on directly to the next step.

*Attempted Synthesis of NVOC-Nitroalanine Cyanomethyl Ester (**8b**)*

Et_3N (35.1 μL, 0.25 mmol) was added to a solution of **7** (90 mg, 0.25 mmol) in neat chloroacetonitrile. The mixture was stirred for 4 h and evaporated to dryness. Flash silica chromatography was used to try to separate the desired product (**8b**) from the side product (**8a**) with a 1:1 hexanes / ethyl acetate solvent system. Purification attempts never yielded pure **8b**, possibly due to degradation to **8a**. ^{1}H-NMR ($CDCl_3$) for **8a** δ 3.98 (s, 3H), 4.01 (s, 3H), 4.89 (s, 2H), 5.60 (s, 2H), 5.95 (m, 1H), 6.44 (s, 1H), 7.01 (s, 1H), 7.74 (s, 1H). ES-MS calculated for **8a** $C_{15}H_{15}N_3O_8$: 365.3, found *m/z* $(M+Na)^+$: 388,

$(M+K)^+$: 404. ES-MS calculated for **8b** $C_{15}H_{16}N_4O_{10}$: 412/3, found *m/z* $(M+Na)^+$: 434.8, $(M+K)^+$: 450.8.

An alternative procedure using a weaker base involved combining **7** (80 mg, 0.2 mmol), Na_2CO_3 (50 mg, 0.5 mmol), and chloroacetonitrile (36 μL, 0.5 mmol) in 5 mL of dry DMSO. The solution was stirred under Ar overnight. The reaction was diluted with 5 mL ethyl acetate and then washed twice with water and twice with brine. Similar ES-MS data found for **8a** and **8b**.

*Preparation of Nitroethylene (**9**)*

2-Nitroethanol (5 mL, 70 mmol) and phthalic anhydride (15.6 g, 105 mmol) were mixed in a distillation setup. As the mixture was heated, the pressure of the distillation setup was kept at 80 mmHg with a VWR automatic vacuum controller (1600B-01). The mixture became a homogeneous yellow liquid at 120ºC; the color changed to orange at 140ºC; the last color change to dark red occurred at 150ºC. Phthalic anhydride would sublimate into the distillation set-up before large amounts of product could be distilled. Often product would be found in the cold finger between the distillation set-up and the vacuum controller. **9** was collected and stored as a 0.8M solution in THF. ^{1}H-NMR ($CDCl_3$) δ 5.90 (d, J = 6.3 Hz, 1H), 6.61 (dd, J = 15 and 2 Hz, 1H), 7.11 (m, J = 15 and 8 Hz, 1H).

*Synthesis of 2-Diphenylmethyleneimino-4-nitro-butanoate tert-Butyl ester (**11**)*

Lithium diisopropylamide (6.8 mL of a 0.5 M solution in THF, 3.4 mmol) was added to a solution of *N*-diphenylmethyleneglycine *tert*-butyl ester (1 g, 3.4 mmol) in THF (6.8 mL) at -78°C. After 1 h, nitroethylene (4.25 mL 0.8 M in THF, 3.4 mmol) was added to the mixture and stirred for an additional 1 h. The reaction mixture was then brought to room temperature and a 1:1 mixture of ethyl acetate and water (20 mL) was added. The organic layer was separated, washed with brine, dried (Mg_2SO_4), and evaporated. The crude product was purified on a flash silica column using a 1:1 ethyl acetate and hexane solvent system to yield 690 mg of pure product (53%): ^{1}H-NMR ($CDCl_3$) δ 1.44 (s, 9H), 2.58 (m, 2H), 4.08 (t, 1H), 4.53 (m, 2H), 7.20 (m, 2H), 7.36 (m, 3H), 7.47 (m, 3H), 7.67 (m, 2H). ES-MS calculated mass for $C_{21}H_{24}N_2O_4$: 368.17, found *m/z* ($M+H^+$): 369.0, ($M+Na^+$): 390.8, ($M+K^+$): 407.0.

*Synthesis of Nitrohomoalanine (**2**)*

2-Diphenylmethyleneimino-4-nitrobutanoate t-Butyl ester was deprotected by addition of 1 N HCl and stirring of the mixture for 15 h at room temperature. The reaction mixture was lyophilized and taken onto the next reaction.

*Synthesis of NVOC-Nitrohomoalanine (**13**)*

Nitrohomoalanine (230 mg, 0.6 mmol) and Na_2CO_3 (132 mg, 1.2 mmol) were dissolved in water (10 mL). To this solution was added NVOC-Cl (171 mg, 0.6 mmol) in

dioxane (10 mL), and the mixture was stirred for 4 h at room temperature. The reaction mixture was evaporated to half of the reaction volume and then diluted with 20 mL of water. The solution was extracted with ether (20 mL) until the organic layer was no longer colored. The aqueous layer was acidified with HCl to a pH of ~ 2 (solution became cloudy) and extracted with dichloromethane until the organic layer was clear. The organic layers were dried and evaporated to yield 209 mg of NVOC-nitrohomoalanine (90%); ES-MS calculated for $C_{14}H_{17}N_3O_{10}$: 387.09, found *m/z* ($M-H^-$): 387.0. Crude product was taken on directly to the next step.

*Synthesis of NVOC-Nitrohomoalanine Cyanomethyl Ester (**14**)*

NVOC-nitrohomoalanine (50 mg, 0.13 mmol) was dissolved in 5 mL of $ClCH_2CN$. Et_3N (18.3 μL, 0.13 mmol) was added and the solution was stirred under Ar for 4 h. The reaction mixture was evaporated and purified on a flash silica column with a 1:1 mixture of ethyl acetate and hexanes to yield 40 mg of product (72%). ^1H NMR (CDCl3) δ 2.95 (m, 2H), 3.18 (m, 2H), 3.96 (s, 3H), 4.02 (s, 3H), 4.33 (m, 1H), 4.96 (d, 2H), 5.56 (d, 2H), 5.72 (d, 1H), 6.91 (s, 1H), 7.72 (s, 1H). ES-MS calculated for $C_{16}H_{18}N_4O_{10}$: 426.10, found *m/z* ($M+Na^+$): 449.0, ($M+K^+$): 465.0.

Synthesis of Nha-dCA

NVOC-nitrohomoalanine cyanomethyl ester (10 mg, 2.3 μmol) was dissolved in 0.5 mL of dry DMF. The dinucleotide dCA was added (10 mg, 8.4 μmol) and the

mixture was stirred under Ar overnight. The reaction mixture was purified by reverse phase HPLC. ES-MS calculated for $C_{33}H_{38}N_{11}O_{22}P_2$: 1005.9, Found *m/z* ($M-H^-$): 1004.2.

A.4 References

(1) Cashin, A. L.; Torrice, M. M.; McMenimen, K. A.; Lester, H. A.; Dougherty, D. A. *Biochemistry* **2007**, *46*, 630–639.

(2) Heitz, F.; Holzwarth, J. A.; Gies, J. P.; Pruss, R. M.; Trumpp-Kallmeyer, S.; Hibert, M. F.; Guenet, C. *European Journal of Pharmacology* **1999**, *380*, 183–195.

(3) Huang, E. S. *Protein Sci*. **2003**, *12*, 1360–1367.

(4) Lu, Z.-L.; Hulme, E. C. *J. Biol. Chem.* **1999**, *274*, 7309–7315.

(5) Shi, L.; Javitch, J. A. *Annual Review of Pharmacology and Toxicology* **2002**, *42*, 437–467.

(6) Mu, T. California Institute of Technology, 2006.

(7) Kelly, T. R., Kim, M.H. *Journal of the American Chemical Society* **1994**, *116*, 7072–7080.

(8) Burgess, V. A.; Easton, C. J. *Australian Journal of Chemistry* **1988**, *41*, 1063–1070.

(9) Coghlan, P. A.; Easton, C. J. *Tetrahedron Letters* **1999**, *40*, 4745–4748.

(10) Byers, J. H.; Baran, R. C.; Craig, M. E.; Jackman, J. T. *Organic Preparations and Procedures International* **1991**, *23*, 373–374.

(11) Krohn, K. *Synthesis-Stuttgart* **1997**, 1115–1127.

(12) Krohn, K.; Küpke, J. *European Journal of Organic Chemistry* **1998**, *1998*, 679–682.

(13) Thiel, W. R.; Krohn, K. *Chemistry—A European Journal* **2002**, *8*, 1049–1058.

(14) Rowley, M.; Leeson, P. D.; Williams, B. J.; Moore, K. W.; Baker, R. *Tetrahedron* **1992**, *48*, 3557–3570.

(15) Vanderwerf, A.; Kellogg, R. M. *Tetrahedron Letters* **1991**, *32*, 3727–3730.

(16) Ranganathan, D., Rao, B., Ranganathan, S., Mehrotra, A.K., Iyengar, R. *Journal of Organic Chemistry* **1980**, *45*, 1185–1189.

(17) Crossley, M. J.; Fung, Y. M.; Potter, J. J.; Stamford, A. W. *Journal of the Chemical Society—Perkin Transactions 1* **1998**, 1113–1121.