Chapter 5. Miscellaneous studies

5.1 Introduction

This chapter contains several miscellaneous studies. These studies represent work that is either in its initial stages or yielded not so interesting results. Two of the studies related in this chapter examine aromatic residues at the ligand-binding site in the Cys loop receptors. These studies were done in collaboration with the Sarah Lummis group at the University of Cambridge. The first study describes investigations of Tyr 198 in the gamma-amino butyric acid C receptor ($GABA_C$). This tyrosine aligns with Trp α149 in the nAChR, and the study of Tyr 198 represents ongoing efforts by our group to characterize the nature of the cation-π interaction in ligand recognition at Cys loop receptors. The second study examines Phe 226 in 5-HT_3R with a series of phenylalanine analogs. This residue aligns with Tyr 190 in the nAChR. This study completes the survey of the aromatic box in the 5-HT_3R. The final study considered in this chapter represents initial efforts to incorporate a backbone thioamide via nonsense suppression methods. This project is now in the capable hands of Lori Lee.

5.2 $GABA_C$ studies

5.2.1 Background

GABA is the main inhibitory neurotransmitter in the nervous system where its action is mediated by three classes of receptors: $GABA_A$, $GABA_B$, and $GABA_C$.[1-3] The $GABA_B$ receptor is a G-protein coupled receptor, while both $GABA_A$ and $GABA_C$ receptors belong to the Cys loop family of ligand-gated ion channels (LGIC). In contrast

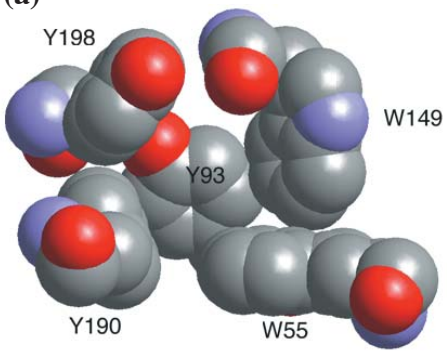
to the nAChR and 5-HT_3R, these are anion-selective ion channels. The $GABA_A$ receptor is the major inhibitory LGIC in the peripheral and central nervous system.[1] The $GABA_C$ receptor is much less prominent and found primarily in the retina, with lower levels in the brain and spinal cord.[1, 4, 5] Three subunits for the $GABA_C$ receptor have thus far been identified (ρ1-ρ3). These can all form functional homomeric receptors, and the ρ1 and ρ2 subunits can coassemble to form heteromeric receptors. [2] The $GABA_C$ receptor displays a distinct physiology and pharmacology from that of $GABA_A$. $GABA_C$ has a higher sensitivity to GABA, does not desensitize, and is insensitive to benzodiazepines, barbiturates, and bicuculline.[6-10]

5.1.2 The ligand-binding site

Based on homology to the nAChR and AChBP, the ligand-binding site in the $GABA_C$ receptor is believed to be located at subunit interfaces with the primary ligand-binding determinants being contributed by residues in five noncontiguous loops, labeled A-E.[1, 3] As with other receptors in the Cys loop family, there is a predominance of aromatic residues at the $GABA_C$ binding site. The x-ray crystal structure of AChBP shows the configuration of these residues to be an 'aromatic box' (Figure 5.1a).[11] In $GABA_C$, four of the five aromatics that comprise the box are conserved (Figure 5.1b). Previous work by our group has demonstrated that the loop B tryptophans in the nAChR (Trp α149) and 5-HT_3R (Trp 183) bind the respective agonists of these receptors via a cation-π interaction.[12, 13] Interestingly, the aligning residue in $GABA_C$ is Tyr 198. In the MOD-1 receptor, another member of the Cys loop family— the loop B residue is also a tyrosine. Here, however, research from our group has demonstrated that the cation-π site is located in loop C at Trp 226.[14] Like the nAChR and 5-HT_3R, this loop C residue is a

tyrosine (Tyr 241) in $GABA_C$. In fact, all residues that form the aromatic box in $GABA_C$ are tyrosines (Figure 5.1b). While the indole ring of tryptophan exhibits the greatest affinity for cations among the standard aromatic residues, the phenolic side chain of tyrosine is also capable of forming strong cation-π interactions.

(a)



(b)

	Loop			
Receptor	**B**	**C1**	**C2**	**D**
nAChR	W149	Y190	Y198	W55
AChBP	W143	Y185	Y192	W53
5-HT_3R	W183	F226	Y234	W90
MOD-1	Y180	Y221	W226	F83
$GABA_C$	Y198	Y241	Y247	Y102

Figure 5.1. Residues at the ligand-binding site in Cys loop LGIC. (a) The aromatic box from the x-ray crystal structure of AChBP. Residue numbering is for muscle nAChR. (b) Alignment of conserved residues in the aromatic box contributed by loops B-D. Red residues represent cation-π binding sites.

In the work presented here a series of fluorinated phenylalanines (4-F-Phe, 3,5-F_2-Phe, 3,4,5-F_3-Phe) is incorporated at position 198 of $GABA_C$ to probe the possible role of this site in binding the ammonium center of GABA via a cation-π interaction (Figure 5.2). The cation-π interaction is a noncovalent binding interaction between the negative electrostatic potential on the face of an aromatic ring and a cation, and as such, is sensitive to perturbation of the ring's electrostatic potential.[15] Thus, the strength of the cation-π interaction can be serially modulated by progressive fluorination of the aromatic ring.

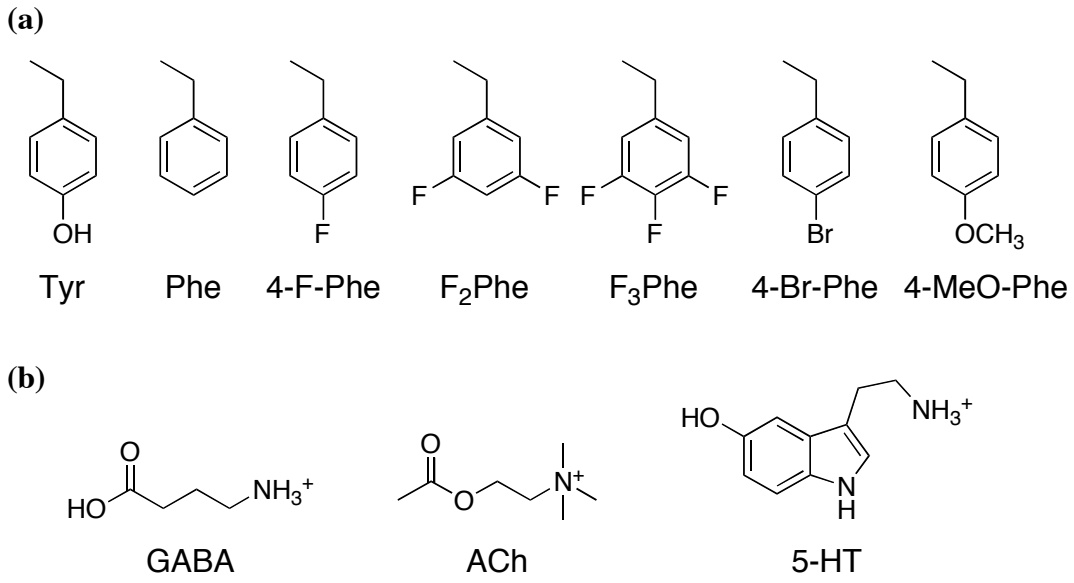


Figure 5.2. (a) Amino acids incorporated at Tyr 198 in $GABA_C$ using nonsense suppression methods. (b) Agonists discussed in the text.

5.1.3 Results and discussion

The unnatural and natural amino acids used in this study were incorporated at position 198 of $GABA_C$ using *in vivo* nonsense suppression methods.[16, 17] The specific $GABA_C$ subtype examined in these studies is the homomeric mouse receptor formed from ρ1 subunits. Initial studies focused on the important controls of nonsense suppression, ensuring no functional receptors are produced in the absence of charged aminoacyl tRNA and rescue of the wild type receptor by reintroduction of tyrosine. Oocytes injected with 198 UAG-containing mRNA only or with UAG-containing mRNA and uncharged tRNA did not show any inducible currents with application of 1 mM GABA, indicating that any readthrough of the mRNA does not produce functional receptors. Injection of oocytes with 198 UAG-containing mRNA and tRNA-Tyr produced receptors with wild type characteristics: EC_{50} values around 2.0 μM and Hill coefficients near 2.5 (Table 5.1).

Incorporation of Phe at 198 leads to a 6-fold increase in EC_{50} (Figure 5.3 and Table 5.1). This is similar to previously published work, where the Phe 198 Tyr mutation yielded an 11-fold increase in EC50.[6] The role of the hydroxyl oxygen was assessed using 4-MeO-Phe and 4-Br-Phe. The unnatural amino acid 4-MeO-Phe maintains an oxygen atom at the 4 position of the ring, which can act as a hydrogen-bond acceptor, but cannot function as a hydrogen bond donor. 4-Br-Phe was used as a measure of the steric requirements of this site. The bromo group is larger than a hydroxyl group, but roughly similar in size to the methoxy group. In addition, 4-Br-Phe and 4-F-Phe bind cations with comparable affinity. Incorporation of 4-MeO-Phe and 4-Br-Phe at 198 resulted in 3- and 4-fold increases in EC_{50}, respectively. Relative to Phe, these mutations show a partial rescue of wild type function, suggesting that both the size of the substituent and the presence of an oxygen at the 4 position of the ring are important.

Before discussing the results from incorporation of the fluorinated phenylalanine series, several important details should be noted. In studies examining tryptophan cation-π sites, the baseline for evaluating the functional effects produced by fluorination is the wild type receptor.[12-14] Tyrosine, however, contains an ionizable hydroxyl group. Fluorination of the phenol ring would lower the pKa of this group and could result in a phenolate anion at this position. Consequently, in considering tyrosine sites, it is necessary to use the fluorinated phenylalanine series which eliminates this complication. The baseline for evaluating the effects of fluorination in these studies, however, is the phenylalanine mutant, and not the wild type residue. This is a reasonable comparison, as phenylalanine and tyrosine bind cations with similar affinity, and any effects arising from

the absence of the hydroxyl group will likely be consistent across the series of phenylalanine derivatives.

Table 5.1. Dose-response data for suppression at 198 in $GABA_C$

Residue	Cation-π binding (kcal/mol)	EC_{50}±SEM (μM)	Hill±SEM
Tyr	26.9	2.1 ± 0.11	2.46 ± 0.25
Phe	27.1	14.0 ± 1.11	2.63 ± 0.43
4-F-Phe	22.0	33.5 ± 4.83	1.9 ± 0.29
F_2Phe	17.1	1631 ± 96	1.95 ± 0.35
F_3Phe	12.9	8550 ± 303	1.55 ± 0.18
4-Br-Phe		9.2 ± 0.08	0.68 ± 0.12
4-MeO-Phe		6.6 ± 0.05	0.66 ± 0.09

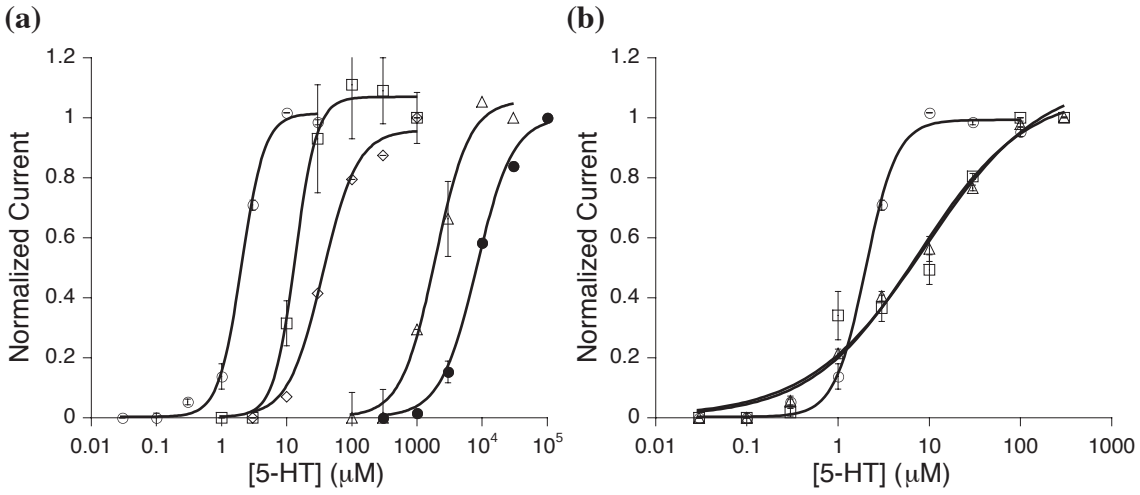


Figure 5.3. Dose-response relations for suppression experiments at 198 in $GABA_C$. (a) Tyr (*open circles*), Phe (*open squares*), 4-F-Phe (*open diamonds*), F_2Phe (*open triangles*), and F_3Phe (*filled circles*). (b) Tyr (*open circles*), 4-Br-Phe (*open squares*), 4-MeO-Phe (*open triangles*)

The dose-response data for incorporation of the fluorinated phenylalanine series shows a clear trend (Figure 5.3 and Table 5.1). Each additional fluorine leads to a concomitant rise in EC_{50}. As in previous work on the nAChR and 5-HT_3R, our measure for the cation-π binding ability of the fluorinated derivatives is the calculated binding

energy (kcal/mol) of a generic probe cation (Na^+) to the corresponding substituted benzene ring.[12-14] Extensive studies of the cation-π interaction establish that *trends* in cation-π binding ability across a series of aromatics are independent of the identity of the cation, justifying the use of a simple probe ion.[15] The calculations for Phe and 4-F-Phe were performed by Sandro Mecozzi and Anthony West[18], and the calculations for 3,5-F_2-Phe and 3,4,5-F_3-Phe were performed by Dennis Dougherty (unpublished results). The fluorination plot (a plot of log EC_{50} versus the calculated cation binding ability of the fluorinated derivatives; see Chapter 2 for details) for these data shows a compelling relationship (Figure 5.4). Over a range of greater than three orders of magnitude in EC_{50}, there is a linear correlation between log EC_{50} (using log EC_{50} to put the EC_{50} data on an energy scale) and the cation-π binding ability of the side chains. This provides substantial evidence that Tyr 198 binds GABA through a cation-π interaction arising from van der Waals contact between the agonist ammonium group and the aromatic side chain.

These findings provide further evidence that the cation-π interaction is a common recognition strategy for agonist binding in the Cys loop receptors. Four members in this family have now been shown to bind their respective ligands through a cation-π interaction.[12-14] Tyr 198 in $GABA_C$ represents the first tyrosine residue to be identified as a cation-π site using fluorinated unnatural amino acids. This study also emphasizes the uniqueness of MOD-1, as $GABA_C$ follows the trend of the nAChR and the 5-HT_3R with the location of the cation-π site in loop B (Figure 5.1). Inspection of the fluorination plot in Figure 5.4b shows that the line for GABA has a similar slope as 5-HT (in both the 5-HT_3R and MOD-1). This supports the idea that, given the large electrostatic component

of the cation-π interaction, primary ammonium groups (Figure 5.2) are more sensitive to electronic perturbation of their aromatic binding partner.[12]

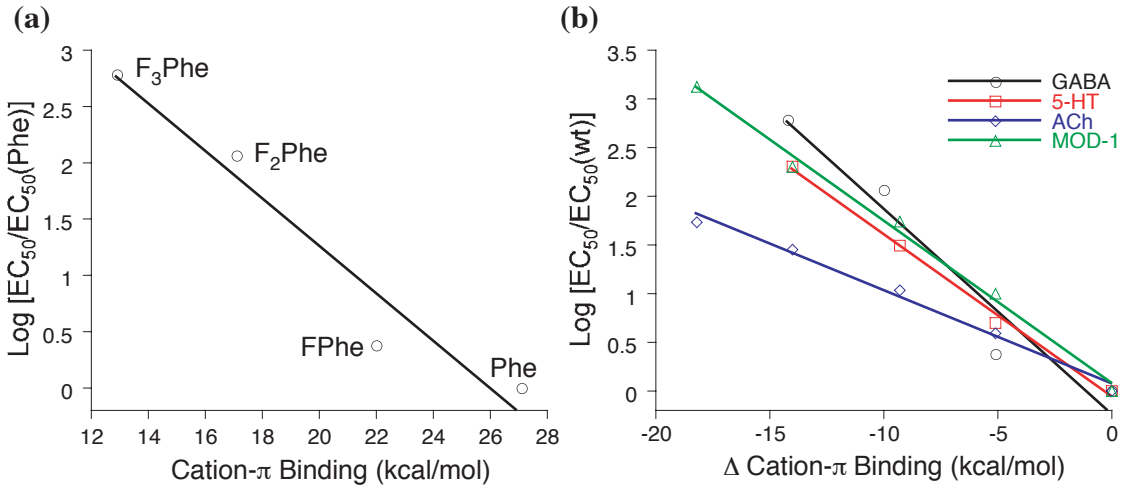


Figure 5.4. Analysis of the cation-π studies in Cys loop LGIC. (a) Fluorination plot for fluoro-Phe series at 198 in $GABA_C$ (the equation for the linear fit is $y = 5.5 - 0.21x$). (b) Fluorination plot for all LGICs with cation-π sites. Two different series of fluorinated aromatics are shown in this plot, thus the *x-axis* reflects the relative binding affinity of each residue in a series. For ACh, 5-HT, and MOD-1 (also 5-HT but different receptor) the data points correspond to fluoro-Trp series and right to left are: Trp, FTrp, F_2Trp, F_3Trp, and F_4Trp. For GABA the plot is for fluoro-Phe series and right to left are: Phe, Fphe, F_2Phe, and F_3Phe.

5.2 Phe 226 in the 5-HT_3R

In the 5-HT_3R, four of the five residues at the aromatic box are conserved (Figure 5.1). In previous work (Chapter 2 and 3), we have examined all but Phe 226.[12, 19] This residue is located in loop C and aligns with Tyr 190 in the nAChR. The functional role of Phe 226 was examined using a series of phenylalanine analogs incorporated via nonsense suppression methods (Figure 5.5).[16, 17]

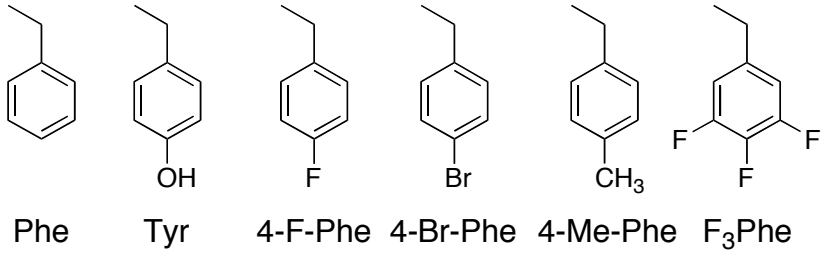


Figure 5.5. Residues used in the study of Phe 226 in 5-HT_3R

Reintroduction of phenylalanine at position 226 produced receptors with an EC_{50} value similar to wild type, 1.6 μM (Table 5.2). Injection of mRNA alone or with uncharged tRNA did not yield functional receptors. The dose-response results for substitution of Phe 226 indicate this is not an overly important position. Incorporation of 4-Me-Phe or 4-Br-Phe leads to a 9-fold increase in EC_{50}. The similarity in results observed for these two unnaturals suggests this increase in EC_{50} is likely a steric effect, and not due to modulation of electrostatic potential on the aromatic ring. This conclusion is supported by the small increase in EC_{50} seen in the 4-F-Phe mutant (4-fold increase.) Interestingly, incorporation of Tyr at 226 leads to a small decrease in EC_{50}. This group of mutations shows Phe 226 is quite tolerant to a variety of substitutions at the 4 position of the aromatic ring.

Table 5.2. Dose-response data for Suppression at 226

Residue	**EC_{50}±SEM (μM)**
Phe	1.6 ± 0.3
Tyr	1.0 ± 0.1
Ala	23.7 ± 5.3
4-Me-Phe	14.3 ± 3.2
4-Br-Phe	14.7 ± 2.4
4-F-Phe	7.1 ± 0.8
F_3-Phe	>300

Two mutations did, however, lead to larger increases in EC_{50}, Ala and F_3-Phe. These lead to 23- and >200-fold increases, respectively. The fact that Ala was less perturbing than F_3Phe is noteworthy in that completely removing the ring has a smaller effect than maintaining the steric size of the ring, but altering the electrostatic potential on the ring. It is also somewhat puzzling that electron–withdrawing groups at the 4 position of the ring are well tolerated, but either the combined effect of three such groups in F_3-Phe or their specific placement on the ring (at positions 3,4, and 5) leads to a large perturbation in receptor function. The latter possibility suggests a specific interaction with one of the ring hydrogens at position 3 or 5.

In the homology model of the 5-HT_3R with 5-HT computationally docked at the binding site, the side chain of Phe 226 is located very near the indole ring of 5-HT (Figure 5.6).[20] The model does not, however, point to any special role for Phe 226. The findings at Phe 226 are quite distinct from what was observed at Tyr 190 in nAChR.[21] Similar substitutions here ablate receptor function or cause large increases in EC_{50}, indicating both the hydroxyl and the aromatic group are critical. These studies demonstrate that there are subtle differences in the functional role of binding-site residues among the receptors in the Cys loop family.

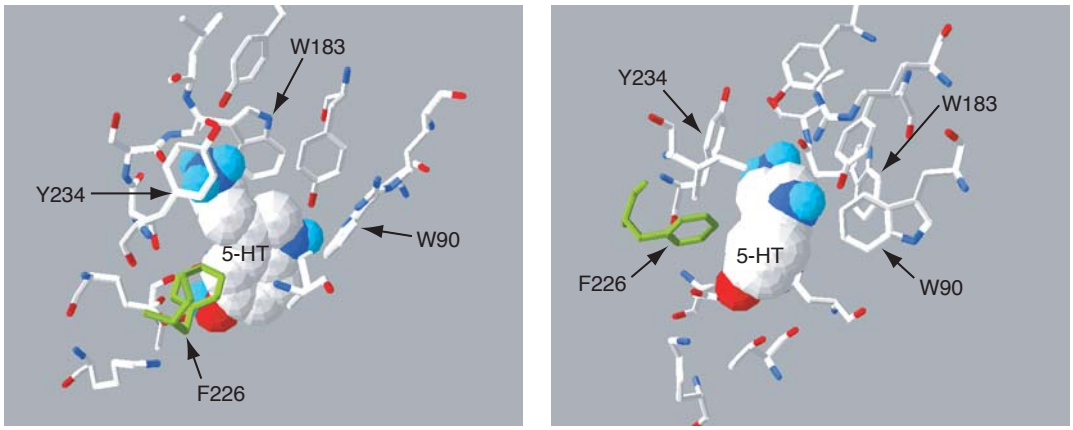


Figure 5.6. Homology model of the 5-HT_3R with 5-HT computationally docked at the ligand-binding site. Phe 226 is shown in green.

5.3 Efforts towards the incorporation of a backbone thioamide

5.3.1 Background

A powerful use of the nonsense suppression method is the introduction of backbone mutations. To date, the method has proven amenable to the incorporation of esters, aza-amides, and oxy-amides into the protein backbone (Figure 5.7a).[22-24] These mutations can be used to probe the hydrogen-bonding properties and local secondary structure at main chain sites. Thus far, the incorporation of backbone mutations has been limited to modifications at the amide nitrogen. Incorporation of a backbone thioamide group represents a potential backbone mutation with modification at the amide oxygen (Figure 5.7b).

The thioamide group has been widely used in the solid-phase synthesis of small peptides for peptidomimetic studies.[25-32] Introduction of a thioamide into the main chain of the protein alters the hydrogen-bonding properties of the protein backbone.[33] Relative to the standard peptide bond, the thioamide carbonyl is a weaker hydrogen-bond acceptor

and the thioamide NH is a stronger hydrogen-bond donor. Thus, in studies of backbone hydrogen-bonding, the thioamide would be a useful complement to main chain ester mutations which remove the hydrogen-bond donor at the amide nitrogen position. In addition, the increased C=S bond length (1.65 Å versus 1.2 Å for C=O) and larger van der Waals radius of the sulfur atom (0.45 Å greater than oxygen) lead to a widening of secondary structure and results in a loss of conformational freedom in the protein backbone.[31, 34, 35] Studies have demonstrated, however, that main chain thioamides do not significantly destabilize α-helices or β-sheets.[26, 27, 31] One particularly attractive feature of the thioamide group is that substitution of oxygen by sulfur in an amide bond leads to significantly red-shifted π–π* and n-π* absorptions, lowering the excitation energy needed for photoisomerization. Recent studies have shown that *cis*/*trans* isomerization of a thioamide group can be readily induced by 250-280 nm light.[36] This latter feature could be a potentially valuable tool for investigating protein conformational changes.

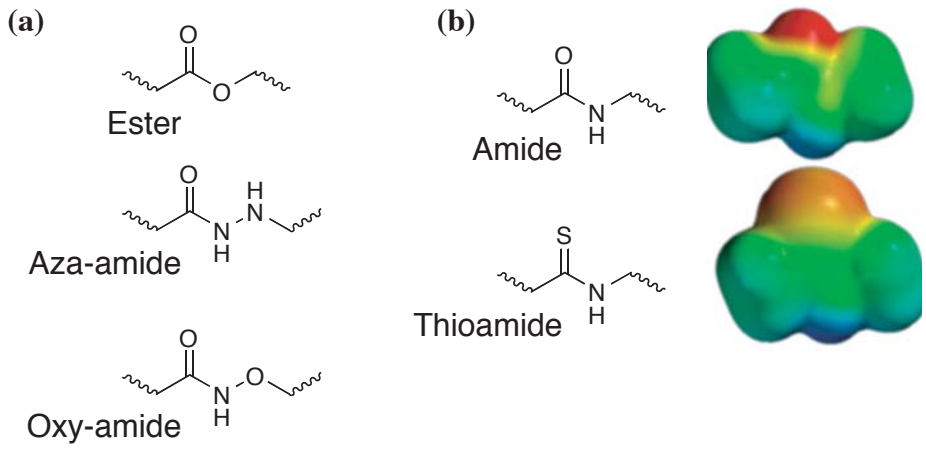


Figure 5.7. Protein backbone mutations. (a) Backbone mutations thus far incorporated by nonsense suppression. (b) Electrostatic potential surfaces for an amide and thioamide, illustrating the relative size of the two groups and the lesser negative potential of sulfur relative to oxygen (red = negative, blue = positive).

5.3.2 Synthetic concerns

Thionation— the conversion of a carbonyl group to a thiocarbonyl— is a widely used synthetic transformation for the preparation of organosulfur compounds. Typically this transformation is accomplished using either phosphorus pentasulfide (P_4S_{10}) or Lawesson's reagent (LR) (Figure 5.8).[37-40] Both of these reagents have a similar reactivity profile. In general, the ease of thionation for a given substrate with these reagents is determined by the electron density at the carbonyl oxygen. Thus, the thionation of amides is usually a fairly facile transformation, while thionation of esters often requires elevated temperatures, longer reaction times, and generally results in poor yields. LR is more commonly used for the thionation of amides as it tends to give higher yields with fewer side reactions.[37] In the case of esters, both reagents have their advantages and disadvantages. The longer reaction times and higher temperatures required for esters means that with either reagent there are significant side reactions. One

advantage of P_4S_{10}, however, is that many of the products from side reactions can be removed through aqueous work-up.[41] It should be noted, however, that because both reagents have highly electrophillic phosphorus species, reactions with either reagent require very dry conditions.

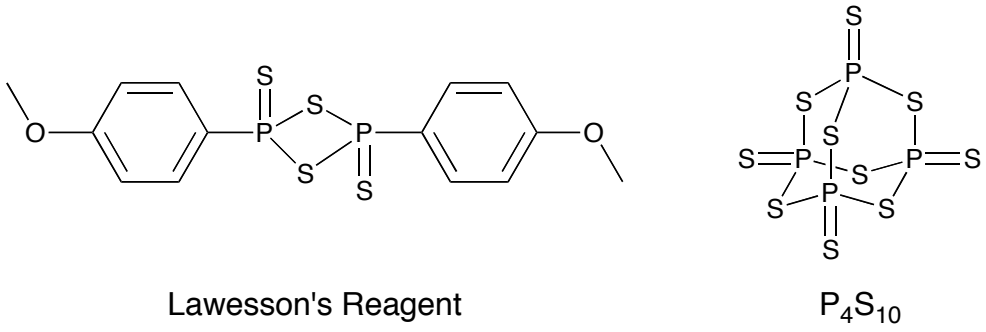
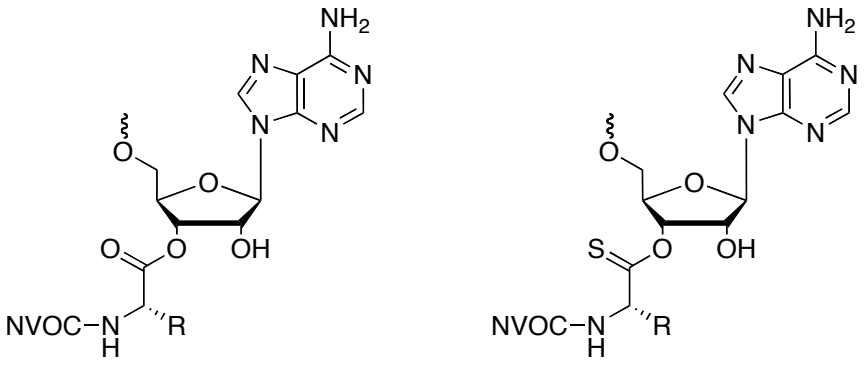


Figure 5.8. Thionation reagents.

Early work on the peptidyl-transfer reaction demonstrated that the ribosome can catalyze the formation of a main chain thioamide in a growing peptide chain.[42] This work used a tRNA where the typical ester linkage between the tRNA and amino acid was replaced by a thionoester— an ester in which the carbonyl oxygen is substituted with sulfur. This study provides evidence for the feasibility of using nonsense suppression to incorporate a backbone thioamide.

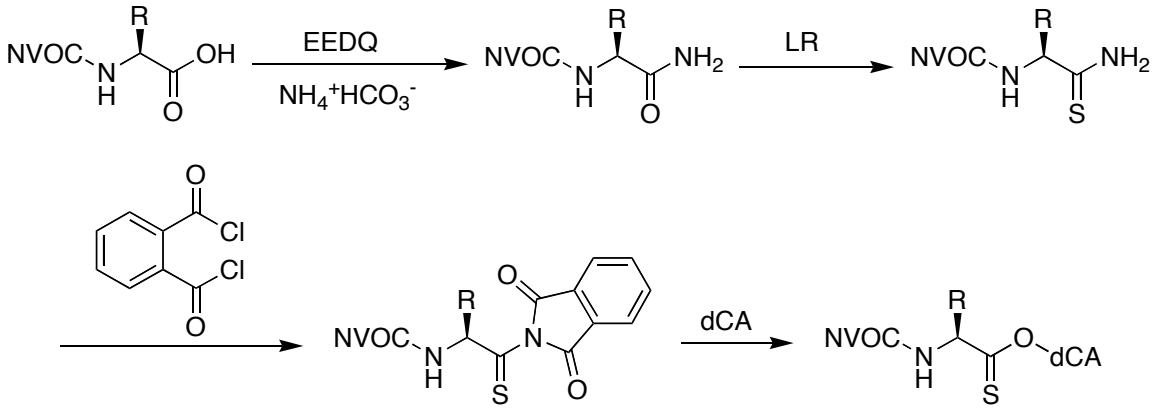
The central challenge in realizing suppression-mediated thioamide incorporation is the synthetic preparation of an amino-thionoacyl dCA, where analogous to the studies above the amino acid and dinucleotide are linked by a thionoester (Figure 5.9). There are two important issues in considering synthetic approaches to this thionoester linkage. First, the thionated residue must be activated in way that is compatible with dCA coupling, and second, thionation of the residue must be synthetically accessible. In solid-phase peptide synthesis thioamides are introduced by replacing the carboxylic acid with

an amide.[28, 29, 43, 44] This is then thionated with LR and activated so that the amine is a good leaving group during the peptide coupling reaction. This strategy avoids having to thionate the activated ester. Adapting this protocol for use with the standard dCA coupling chemistry used in preparation of aminoacyl dCA represents one possible approach to generating aminoacyl dCA with a thionoester linkage (Scheme 5.1).[43, 44] This method, however, would require the development of new dCA coupling chemistry. A second approach to this problem is to thionate the activated cyanomethyl ester and then couple the thionated ester with dCA (Scheme 5.2). This route uses the standard dCA coupling chemistry, but has the drawback of having to thionate an ester.

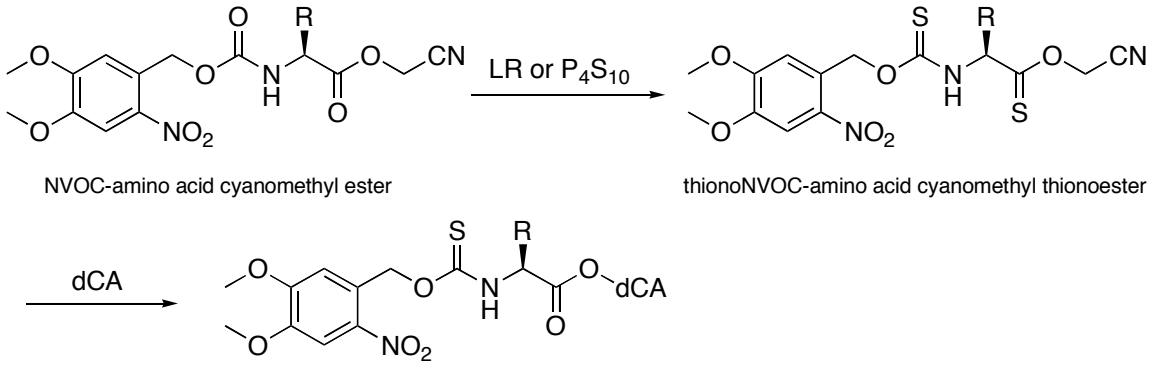


Aminoacyl dCA Amino-thionoacyl dCA

Figure 5.9. Schematic illustrating the ester linkage between the amino acid and the dinucleotide dCA and the thionoester linkage with dCA that is necessary for incorporation of a thioamide via nonsense suppression methods.



Scheme 5.1. Synthetic route to amino thionoacyl dCA, adapted from strategies used in the solid-phase synthesis of peptides containing thioamides.



Scheme 5.2. Synthetic route to amino thionoacyl dCA, using standard dCA coupling chemistry.

5.3.3 Experiments

5.3.3.1 Synthesis of thionoNVOC-Pro cyanomethyl thionoester

Initial attempts to synthesize amino thionoacyl dCA followed the route shown in Scheme 5.2. In the end, it was decided that remaining with the standard chemistry used in nonsense suppression represented a better strategy. It is also a shorter route, as it requires only one additional step to be added to the standard amino acid activation and dCA coupling protocol. It should be noted that carbamates are generally quite reactive

with LR, and thus, it is anticipated that the NVOC carbonyl will also be thionated.[37] This does not seem to be problematic, as the thionated species should not substantially perturb the photolysis reaction. The first thionation reaction used NVOC-Pro cyanomethyl ester as the substrate and LR as the thionating reagent. Proline was selected for the potential use of the thiono-analog in studies of the M2-M3 loop (Chapter 4). NVOC-Pro cyanomethyl ester was synthesized in the usual manner.

NVOC-Pro cyanomethyl ester (150 mg, 0.39 mmol) was placed in an oven-dried flask. To this was added 2 mL of dry toluene and 3.6 molar equivalents of LR (568 mg, 14 mmol). The reaction was run under positive argon pressure, heated to reflux in an oil bath, and stirred. The reaction was stopped after 12 hours and applied directly to a flash chromatography column (silica, hexanes/ethyl acetate 1:1). NMR analysis of the column fractions showed no product, but did reveal that the column fractions contained multiple compounds. The diagnostic assay for thionation of an ester is ^{13}C NMR.[30] Thionation leads to ~30 ppm upfield shift in the carbonyl carbon signal. A very typical ^{13}C chemical shift for the carbonyl carbon in a cyanomethyl ester is ~170 ppm. Thus it was expected the product would show a peak near 200 ppm. Thionation of an ester does not typically lead to diagnostic chemical shifts in the ^{1}H NMR spectrum.

The reaction was repeated using identical conditions. This time, however, the reaction was monitored by reverse-phase HPLC and UV spectroscopy. Purification was carried out using semi-preparative reverse-phase HPLC. The chromatogram showed two peaks with significant 350 nm absorption (NVOC group). One matched the elution profile of NVOC-Pro cyanomethyl ester. Fractions corresponding to the other peak were isolated and lyophilized yielding a yellow/brown powder (24 mg, 14% yield). ^{1}H NMR

(300 MHz, $CDCl_3$) δ = 1.95-2.04 (4H, m), 2.21-2.29 (2H, m), 3.88 (3H, s) 3.90 (3H, s), 4.33-4.37 (1H, m), 4.97 (2H, q), 5.45 (2H, dd), 6.88 (1H, s), 7.63 (1H, s). ^{13}C NMR (75 MHz, $CDCl_3$) δ = 25.4, 30.4, 30.8, 47.3, 56.1, 57.4, 60.5, 65.7, 69.8, 108.3, 110.2, 128.3, 140.8, 148.6, 154.3, 156.2, 172.3. MS Calcd for $C_{17}H_{19}N_3O_6S_2$ 425.48. Found: (ESI^+) 426.8 $[M+H^+]^+$.

The mass spectroscopy data correspond to thionoNVOC-Pro cyanomethyl thionoester, with thionation at both the ester carbonyl and the carbamate carbonyl as was expected. Interestingly, the ^{13}C data do not show a shift in the carbonyl carbon peaks for the ester or the carbamate, 172.3 and 156.2 ppm, respectively. The corresponding peaks in NVOC-Pro cyanomethyl ester for the ester and carbamate carbons are 171.9 and 155.4 ppm, respectively. The ^{13}C and ^1H NMR spectra do show differences in the chemical shifts between the two compounds, but the origin of these differences was not able to be determined. Ultimately the spectral data are ambiguous as to the identity of the isolated product. The mass spectroscopy analysis indicates it is thionoNVOC-Pro cyanomethyl thionoester, but the ^{13}C NMR data do not show the diagnostic shifts. Despite this uncertainty, the product was taken on to the dCA coupling step. For comparison the spectral data for NVOC-Pro cyanomethyl ester are shown below: ^1H NMR (300 MHz, $CDCl_3$) δ = 1.68-1.88 (4H, m), 2.07-2.15 (2H, m), 3.69 (3H, s) 3.77 (3H, s), 4.20-4.23 (1H, m), 4.59 (2H, q), 5.25 (2H, dd), 6.74 (1H, s), 7.42 (1H, s). ^{13}C NMR (75 MHz, $CDCl_3$) δ = 24.7, 29.5, 31.0, 36.3, 49.8, 54.3, 56.2, 58.8, 64.8, 110.1, 114.2, 128.7, 139.8, 148.4, 154.3, 155.4, 171.9. MS Calcd for $C_{17}H_{19}N_3O_8$ 393.35. Found: (ESI^+) 394.4 $[M+H^+]^+$

5.3.3.2 dCA coupling

Coupling of the speculative thionoNVOC-Pro cyanomethyl thionoester to dCA was done in the usual manner. To a dry flask was added thionoNVOC-Pro cyanomethyl thionoester (24 mg, 0.056 mmol), dCA (20 mg), and 0.8 mL of dry DMF, and the reaction was stirred under argon. The reaction was monitored by reverse-phase HPLC. After 6 hours no product formation was seen, so tetrabutylammonium acetate (10 mg) was added to the reaction. The reaction immediately turned scarlet red. After 48 hours no product formation was observed and the chromatogram for the reaction showed a multitude of side reactions.

5.3.3.3 Attempts using microwave irradiation

The reactions detailed above revealed several problems with the initial strategy. The reactions showed a significant number of side reactions. The use of excess LR as is required in the thionation of esters made the purification problematic by either flash chromatography (insufficient purification) or reverse-phase HPLC (time consuming), and ultimately, the results were ambiguous.[37] Recently, several groups have reported facile thionation of esters using solvent-free microwave reactions.[45, 46] These studies reported high yields, required only minutes to run, and were able to thionate recalcitrant substrates. These studies also adapted a procedure commonly used in P_4S_{10} thionations, where it has been noted that inclusion of the scavenger hexamethyl-disiloxane (HMDO) in the reaction mix reduces the number of side products and improves the yield.[38, 41]

For the solvent-free microwave-assisted reactions described below, NVOC-Gly cyano methyl ester was used as the substrate. This was done to simplify the NMR

spectra and for potential proof of principle experiments involving the *Shaker* potassium channel (discussed in 5.3.4 Future directions).

In the first reaction, NVOC-Gly cyanomethyl ester (100 mg, 0.28 mmol) and LR (183 mg, 0.45 mmol) were placed in a test tube and thoroughly mixed with a spatula. The test tube was placed in an alumina bath (alumina in a beaker) and irradiated with an unmodified household microwave at maximum wattage (1000 W) for 3 minutes (continuous radiation). This reaction yielded black charred material. The second reaction was set up in an identical manner and irradiated for 5 minutes (10 x 30 s). Following irradiation, the reaction mix was dissolved in methylene chloride and loaded on a flash chromatography column (silica, hexanes/ethyl acetate 1:1). Neither product nor starting material was isolated. In the third attempt, HMDO (73 mg, 95 μL, 0.45 mmol) was included in the reaction mix and the reaction was irradiated for 5 min. (10 x 30 s). Again, neither products nor starting materials were isolated by flash chromatography.

5.3.4 Future directions

There are several possible directions that can be taken to address the problems encountered in trying to synthesize amino thionoacyl dCA. The first is the use of model compounds. This would allow a better estimation of the proper reaction conditions. As noted in the introductory section, thionation reactions with LR or P_4S_{10} are very sensitive to water. It may be that the conditions were not dry enough. In addition, the reactions were run with 3.6 molar equivalents of LR. This excess— although standard in thionations of esters— certainly must lead to more side products as well as making purification in general more difficult.[37, 39] It would also be worthwhile to include HMDO

in the standard LR reactions, and reactions using P_4S_{10} should be considered. If none of these steps prove sufficient, then the route illustrated in Scheme 5.1 offers an alternative that avoids having to thionate an ester.

Once the synthetic issues are worked out, the early studies on the peptidyl-transfer reaction indicate this mutation is compatible with the ribosome and thus, should be amenable to incorporation by nonsense suppression methods. Once incorporated into the protein, backbone thioamides can be readily cleaved by treatment with trifluoracetic acid.[47] The mechanism for this cleavage proceeds in a similar manner to the Edman degradation. This would provide a direct means for verifying the successful incorporation of a backbone thioamide, as the full-length protein and the cleaved protein would show different gel mobilities. Finally, a nice proof of principle experiment would be the incorporation of a thionoGly at the selectivity filter of the *Shaker* potassium channel. The selectivity filter in potassium channels is composed of several backbone carbonyls that point into the channel lumen. The carbonyl oxygen atoms serve to coordinate the potassium ion as it enters the channel. Thus, replacement of the oxygens with the less electronegative sulfur atom should alter the ion selectivity and permeation properties of these channels.

5.4 References

1. Bormann, J., The 'ABC' of GABA receptors. *Trends in Pharmacological Sciences* **2000,** 21, (1), 16-19.
2. Enz, R., GABA(C) receptors: A molecular view. *Biological Chemistry* **2001,** 382, (8), 1111-1122.
3. Jentsch, T. J.; Stein, V.; Weinreich, F.; Zdebik, A. A., Molecular structure and physiological function of chloride channels. *Physiological Reviews* **2002,** 82, (2), 503-568.
4. Cutting, G. R.; Lu, L.; Ohara, B. F.; Kasch, L. M.; Montroserafizadeh, C.; Donovan, D. M.; Shimada, S.; Antonarakis, S. E.; Guggino, W. B.; Uhl, G. R.; Kazazian, H. H., Cloning of the Gamma-Aminobutyric-Acid (Gaba) Rho-1 Cdna - a Gaba Receptor Subunit Highly Expressed in the Retina. *Proceedings of the National Academy of Sciences of the United States of America* **1991,** 88, (7), 2673-2677.
5. Zheng, W.; Xie, W. R.; Zhang, J. H.; Strong, J. A.; Wang, L.; Yu, L.; Xu, M.; Lu, L., Function of gamma-aminobutyric acid receptor/channel rho(1) subunits in spinal cord. *Journal of Biological Chemistry* **2003,** 278, (48), 48321-48329.

6. Amin, J. a. W., D.S., Homomeric r1 GABA channels: activation mechanisms and domains. *Receptors and Channels* **1994,** 2, 227-236.
7. Chang, Y. C.; Covey, D. F.; Weiss, D. S., Correlation of the apparent affinities and efficacies of gamma-aminobutyric acid(C) receptor agonists. *Molecular Pharmacology* **2000,** 58, (6), 1375-1380.
8. Martinez-Torres, A.; Demuro, A.; Miledi, R., GABA rho 1/GABA(A)alpha 1 receptor chimeras to study receptor desensitization. *Proceedings of the National Academy of Sciences of the United States of America* **2000,** 97, (7), 3562-3566.
9. Wotring, V. E.; Chang, Y. C.; Weiss, D. S., Permeability and single channel conductance of human homomeric rho 1 GABA(C) receptors. *Journal of Physiology-London* **1999,** 521, (2), 327-336.
10. Zhang, D. X.; Pan, Z. H.; Awobuluyi, M.; Lipton, S. A., Structure and function of GABA(c) receptors: a comparison of native versus recombinant receptors. *Trends in Pharmacological Sciences* **2001,** 22, (3), 121-132.
11. Brejc, K.; van Dijk, W. J.; Klaassen, R. V.; Schuurmans, M.; van der Oost, J.; Smit, A. B.; Sixma, T. K., Crystal structure of an ACh-binding protein reveals the ligand-binding domain of nicotinic receptors. *Nature* **2001,** 411, (6835), 269-276.
12. Beene, D. L.; Brandt, G. S.; Zhong, W. G.; Zacharias, N. M.; Lester, H. A.; Dougherty, D. A., Cation-pi interactions in ligand recognition by serotonergic (5-HT3A) and nicotinic acetylcholine receptors: The anomalous binding properties of nicotine. *Biochemistry* **2002,** 41, (32), 10262-10269.
13. Zhong, W. G.; Gallivan, J. P.; Zhang, Y. N.; Li, L. T.; Lester, H. A.; Dougherty, D. A., From ab initio quantum mechanics to molecular neurobiology: A cation-pi binding site in the nicotinic receptor. *Proceedings of the National Academy of Sciences of the United States of America* **1998,** 95, (21), 12088-12093.
14. Mu, T. W.; Lester, H. A.; Dougherty, D. A., Different binding orientations for the same agonist at homologous receptors: A lock and key or a simple wedge? *Journal of the American Chemical Society* **2003,** 125, (23), 6850-6851.
15. Ma, J. C.; Dougherty, D. A., The cation-pi interaction. *Chemical Reviews* **1997,** 97, (5), 1303-1324.
16. Nowak, M. W.; Kearney, P. C.; Sampson, J. R.; Saks, M. E.; Labarca, C. G.; Silverman, S. K.; Zhong, W.; Thorson, J.; Abelson, J. N.; Davidson, N.; Schultz, P. G.; Dougherty, D. A.; Lester, H. A., Nicotinic Receptor-Binding Site Probed with Unnatural Amino-Acid-Incorporation in Intact-Cells. *Science* **1995,** 268, (5209), 439-442.
17. Nowak, M. W.; Gallivan, J. P.; Silverman, S. K.; Labarca, C. G.; Dougherty, D. A.; Lester, H. A., In vivo incorporation of unnatural amino acids into ion channels in Xenopus oocyte expression system. In *Ion Channels, Pt B* **1998**, 293, 504-529.
18. Mecozzi, S.; West, A. P.; Dougherty, D. A., Cation-pi interactions in simple aromatics: Electrostatics provide a predictive tool. *Journal of the American Chemical Society* **1996,** 118, (9), 2307-2308.
19. Beene, D. L.; Price, K. L.; Lester, H. A.; Dougherty, D. A.; Lummis, S. C. R., Tyrosine residues that control binding and Gating in the 5-hydroxytryptamine(3) receptor revealed by unnatural amino acid mutagenesis. *Journal of Neuroscience* **2004,** 24, (41), 9097-9104.
20. Reeves, D. C.; Sayed, M. R. F.; Chau, P. L.; Price, K. L.; Lummis, S. C. R., Prediction of 5-HT3 receptor agonist-binding residues using homology modeling. *Biophysical Journal* **2003,** 84, (4), 2338-2344.
21. Kearney, P. C.; Nowak, M. W.; Zhong, W.; Silverman, S. K.; Lester, H. A.; Dougherty, D. A., Agonist binding site of the nicotinic acetylcholine receptor: Tests with novel side chains and with several agonists. *Molecular Pharmacology* **1996,** 50, (5), 1401-1412.
22. Koh, J. T.; Cornish, V. W.; Schultz, P. G., An experimental approach to evaluating the role of backbone interactions in proteins using unnatural amino acid mutagenesis. *Biochemistry* **1997,** 36, (38), 11314-11322.
23. Eisenhauer, B. M.; Hecht, S. M., Site-specific incorporation of (aminooxy)acetic acid into proteins. *Biochemistry* **2002,** 41, (38), 11472-11478.
24. Killian, J. A.; Van Cleve, M. D.; Shayo, Y. F.; Hecht, S. M., Ribosome-mediated incorporation of hydrazinophenylalanine into modified peptide and protein analogues. *Journal of the American Chemical Society* **1998,** 120, (13), 3032-3042.
25. Levesque, G.; Arsene, P.; Fanneau-Bellenger, V.; Pham, T. N., Protein thioacylation. 1. Reagents design and synthesis. *Biomacromolecules* **2000,** 1, (3), 387-399.
26. Miwa, J. H.; Patel, A. K.; Vivatrat, N.; Popek, S. M.; Meyer, A. M., Compatibility of the thioamide functional group with beta-sheet secondary structure: Incorporation of a thioamide linkage into a beta-hairpin peptide. *Organic Letters* **2001,** 3, (21), 3373-3375.

27. Miwa, J. H.; Pallivathucal, L.; Gowda, S.; Lee, K. E., Conformational stability of helical peptides containing a thioamide linkage. *Organic Letters* **2002,** 4, (26), 4655-4657.
28. Shalaby, M. A.; Grote, C. W.; Rapoport, H., Thiopeptide synthesis. alpha-amino thionoacid derivatives of nitrobenzotriazole as thioacylating agents. *Journal of Organic Chemistry* **1996,** 61, (25), 9045-9048.
29. Shalaby, M. A.; Rapoport, H., A general and efficient route to thionoesters via thionoacyl nitrobenzotriazoles. *Journal of Organic Chemistry* **1999,** 64, (3), 1065-1070.
30. Sifferlen, T.; Rueping, M.; Gademann, K.; Jaun, B.; Seebach, D., beta-Thiopeptides: Synthesis, NMR solution structure, CD spectra, and photochemistry. *Helvetica Chimica Acta* **1999,** 82, (12), 2067-2093.
31. Tran, T. T.; Zeng, J.; Treutlein, H.; Burgess, A. W., Effects of thioamide substitutions on the conformation and stability of alpha- and 3(10)-helices. *Journal of the American Chemical Society* **2002,** 124, (18), 5222-5230.
32. Zacharie, B.; Lagraoui, M.; Dimarco, M.; Penney, C. L.; Gagnon, L., Thioamides: Synthesis, stability, and immunological activities of thioanalogues of Imreg. Preparation of new thioacylating agents using fluorobenzimidazolone derivatives. *Journal of Medicinal Chemistry* **1999,** 42, (11), 2046-2052.
33. Aleman, C., On the ability of modified peptide links to form hydrogen bonds. *Journal of Physical Chemistry A* **2001,** 105, (27), 6717-6723.
34. Artis, D. R.; Lipton, M. A., Conformations of thioamide-containing dipeptides: A computational study. *Journal of the American Chemical Society* **1998,** 120, (47), 12200-12206.
35. Tran, T. T.; Treutlein, H.; Burgess, A. W., Conformational analysis of thiopeptides: derivation of Sp(2) sulfur parameters for the CFF91 force field. *Journal of Computational Chemistry* **2001,** 22, (10), 1010-1025.
36. Helbing, J.; Bregy, H.; Bredenbeck, J.; Pfister, R.; Hamm, P.; Huber, R.; Wachtveitl, J.; De Vico, L.; Olivucci, M., A fast photoswitch for minimally perturbed peptides: Investigation of the trans -> cis photoisomerization of N-methylthioacetamide. *Journal of the American Chemical Society* **2004,** 126, (28), 8823-8834.
37. Cava, M. P.; Levinson, M. I., Thionation Reactions of Lawesson Reagents. *Tetrahedron* **1985,** 41, (22), 5061-5087.
38. Curphey, T. J., Thionation of esters and lactones with the reagent combination of phosphorus pentasulfide and hexamethyldisiloxane. *Tetrahedron Letters* **2002,** 43, (3), 371-373.
39. Pedersen, B. S.; Scheibye, S.; Nilsson, N. H.; Lawesson, S. O., Studies on Organophosphorus Compounds .10. Syntheses of Thioketones. *Bulletin Des Societes Chimiques Belges* **1978,** 87, (3), 223-228.
40. Sudalai, A.; Kanagasabapathy, S.; Benicewicz, B. C., Phosphorus pentasulfide: A mild and versatile catalyst-reagent for the preparation of dithiocarboxylic esters. *Organic Letters* **2000,** 2, (20), 3213-3216.
41. Curphey, T. J., Thionation with the reagent combination of phosphorus pentasulfide and hexamethyldisiloxane. *Journal of Organic Chemistry* **2002,** 67, (18), 6461-6473.
42. Victorova, L. S.; Kotusov, V. V.; Azhaev, A. V.; Krayevsky, A. A.; Kukhanova, M. K.; Gottikh, B. P., Synthesis of Thioamide Bond Catalyzed by Escherichia-Coli Ribosomes. *Febs Letters* **1976,** 68, (2), 215-218.
43. Josse, O.; Labar, D.; Marchand-Brynaert, J., A convenient synthesis of ethyl 3-aminopropanedithioate (beta- alanine ethyl dithioester). *Synthesis-Stuttgart* **1999**, (3), 404-406.
44. Brain, C. T.; Hallett, A.; Ko, S. Y., Thioamide synthesis: Thioacyl-N-phthalimides as thioacylating agents. *Journal of Organic Chemistry* **1997,** 62, (12), 3808-3809.
45. Varma, R. S.; Kumar, D., Microwave-accelerated solvent-free synthesis of thioketones, thiolactones, thioamides, thionoesters, and thioflavonoids. *Organic Letters* **1999,** 1, (5), 697-700.
46. Filippi, J. J.; Fernandez, X.; Lizzani-Cuvelier, L.; Loiseau, A. M., Solvent-free thionation of gamma-lactones under microwave irradiation. *Tetrahedron Letters* **2003,** 44, (35), 6647-6650.
47. Miwa, J. H.; Margarida, L. A.; Meyer, A. E., Improved acidolytic deprotection conditions for the Fmoc-based solid-phase synthesis of thioxo peptides. *Tetrahedron Letters* **2001,** 42, (41), 7189-7191.