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Design and Synthesis of a Novel Isoxazoline as a Potential PAM-Agonist

Josselyn Roosenberg
Andrews University, josselyn@andrews.edu

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Honors Thesis

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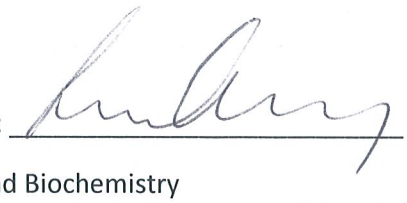
Advisor: Dr. Lisa Ahlberg

Primary Advisor Signature: 

Department: Chemistry and Biochemistry

Abstract

Progression of Alzheimer's disease is associated with a loss of M1 receptor activation in the brain. However, a lack of clinical success following attempts to activate the M1 receptor at the orthosteric site has contributed to a transition towards the allosteric pocket of the receptor. Here, positive allosteric modulators (PAMs) interact to potentiate the acetylcholine response. Recent research proposes that optimization of PAM activity at the expense of intrinsic agonism may posit a means to limit adverse side effects. Therefore, this project proposes a design and synthesis of a novel isoxazoline as a potent PAM agent with weak intrinsic agonism.

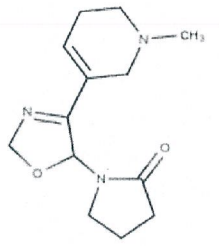
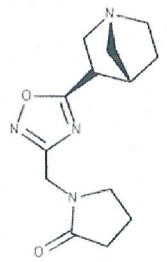
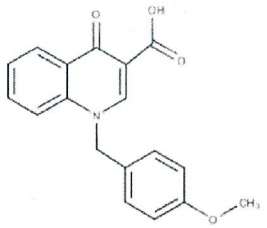
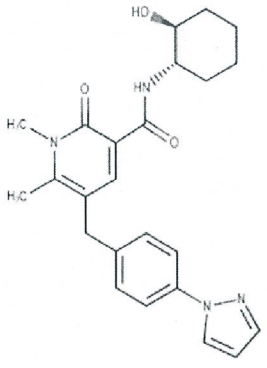
Introduction

Alzheimer's Disease is a neurogenerative disease characterized by memory loss and deterioration of cognitive abilities. In fact, the population of those diagnosed with the disease is only predicted to grow substantially in the coming years.[1] Whereas there is currently no known cure or preventative for the disease, much attention has been devoted to the role of the cholinergic system in disease progression. A central hallmark of Alzheimer's disease includes cholinergic hypofunction following degeneration of cholinergic neurons that project into the cortex and hippocampus of the brain.[2] The cholinergic hypothesis proposes that the loss of pre-synaptic cholinergic activity has a major role in the progressive cognitive impairment associated with Alzheimer's disease (AD).[2] Therefore, activation of the cholinergic system could potentially reverse the deficits characteristic of AD.[3] Attempts to activate the brain's cholinergic system have focused on the M1 muscarinic acetylcholine receptor (mAChR) for its association with the success of cholinergic drugs on cognition in the context of AD.[4] Traditional approaches to selectively activate the M1 receptor have employed agonistic compounds that directly induce the endogenous acetylcholine response. However, these attempts have yielded little success with agonistic drugs halted in clinical trials as a result of adverse cholinergic effects, attributed to non-selective muscarinic receptor activation.[5]

As a result, research has shifted its focus to the allosteric site of the M1 receptor, introducing a new class of compounds known as positive allosteric modulatory (PAM) agonists.[6] The allosteric site is thought to lie above the orthosteric site, offering a location at which specific compounds can bind to promote the acetylcholine response. PAM-agonists are compounds that can function as an agonist, interacting directly at the orthosteric site to promote receptor activation, or as a positive allosteric modulator (PAM), binding at the allosteric site to promote receptor activation in the presence of acetylcholine. However, examination of the pharmacology and safety of recent literature PAM-agonists have produced results reflective of those observed with M1 mAChR agonists.[6,7] For example, single dose escalation studies of PAM-agonists on dogs have resulted in adverse cholinergic events, including convulsion. Recent research hypothesizes that these side effects may stem from the agonistic ability of PAM-agonists and propose that optimization of PAM activity at the expense of intrinsic agonism may posit a means to limit adverse side effects.[8] Therefore, this project attempts to merge recent literature findings, in regards to the structural changes that govern PAM activity vs. agonistic activity, in the design of a novel isoxazoline derivative as a potential PAM-agonist. An examination of structure activity relationship (SAR) data at the allosteric site and incorporation of the structural motifs underlying potent PAM activity and weak agonism, allow for the construction of an isoxazoline compound of interest. Furthermore, this project proposes a synthetic route for the proposed isoxazoline compound, incorporating a 1,3 dipolar cycloaddition reaction.

Design of a Novel Isoxazoline Compound

The design of a novel PAM agonist involves the examination of the literature compounds displayed in Table 1 for those structural characteristics that govern PAM activity as opposed to intrinsic agonism. Table 1 displays the structure of seven lead compounds, noting their key features and those conclusions useful in the design of a novel structure as a potential PAM-agonist.

Compound Number	Compound Structure	Reference	Key Features	Conclusions
1		(10)	- isoxazole core, six-membered cyclic top, pyrrolidone tail - three membered structure	- isoxazoline derivative gives selective M1 mAChR agonism - docking studies at orthosteric site fail to justify selectivity
2		(11)	- oxadiazole core, six-membered cyclic top, pyrrolidone tail - three membered structure	- reinforces use of an isoxazoline structure - docking studies suggest selectivity may be a result of preferential allosteric binding
3		(7)	- aromaticity of core and pendant regions	- first PAM introduced in literature - structure suggests aromaticity as key to PAM activity
4		(6)	- pyridone core, cyclohexanol top, pyrazole tail - intramolecular hydrogen bond	- reduction of core retains PAM activity - pryidone core and aromatic tail pi stack with surrounding residues - intramolecular hydrogen bond promotes active conformation

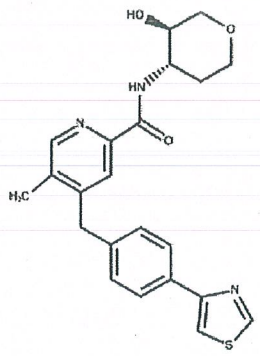
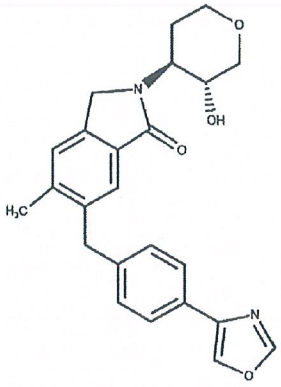
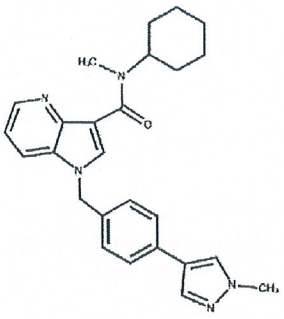
5		(6)	- pyridine core, tetrahydrofuran top, thiazole tail	- pyridine core provided favorable CNS penetration - tetrahydrofuran ring decreased drug liver clearance - thiazole tail increased receptor activity
6		(7)	- lactam core, tetrahydrofuran top, oxazole tail - intramolecular hydrogen bond as a covalent constraint	- methyl group of fused bicyclic system orients benzyl tail - benzyl oxazole tail contributed towards low clearance, a long half-life, and CNS penetrance
7		(8)	- azaindole core, cyclohexyl top, pyrazole tail	- structure provided significantly lower intrinsic efficacy than first published PAM - top region tolerated a high degree of variability, such as a lack of heteroatom presence - core simplification provided lower intrinsic efficacy

Table 1: An analysis of literature compounds

The design of a novel PAM-agonist begins with an examination of Compound 3 in Table 1, the first PAM compound introduced in literature. Compound 3 illustrates an important structural scaffold: a three membered structure containing top, core, and tail portions. This structural motif is preserved within the other PAM literature compounds explored in addition to the aromaticity of the core and pendant structures. Consequently, the design of a novel PAM-agonist (Figure 1) retains these characteristics, incorporating aromaticity and preserving the top, core, and tail structural organization.

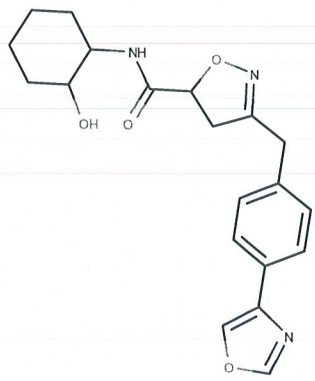


Figure 1: Proposed Isoxazoline Structure

As Figure 1 illustrates, an isoxazoline ring was chosen as the core of the designed structure. The isoxazoline skeleton, containing oxygen and nitrogen heteroatoms in addition to a double bond (Figure 1), has demonstrated significant cytotoxic, antiviral, antimicrobial, and anti-inflammatory uses among other applications.[9] The isoxazoline ring and related structures were first introduced to the discussion of selective M1 mAChR activation through the work of Methusamy et al. and Huang et al. in Compounds 1 and 2 in Table 1. Compounds 1 and 2 were not explored as PAM-agonists but as selective M1 mAChR agonists. However, docking studies performed by Huang and colleagues at the M1 mAChR orthosteric site failed to justify the selectivity of lead isoxazoline Compound 1.[10] Likewise, Muthusamy and colleagues failed to justify the selectivity of Compound 2 by examining the interaction of the compound with the receptor at the orthosteric site.[11] Instead, they proposed that selectivity was a result of preferential binding at the allosteric site of the M1 mAChR. Design of the novel isoxazoline builds on these discoveries, recognizing the potential of an isoxaoline to interact selectively at the allosteric site of the M1 mAChR. Furthermore, the literature compounds displayed in Table 1 affirm the use of a cyclic core, containing electronegative atoms such as oxygen or nitrogen. Davoren et al. demonstrate that these characteristics of the core allow for pi stacking with surrounding amino acids at the allosteric site and induce the correct conformation of the tail pendant.[6]

Muthusamy et al. also note that insertion of a methylene linker between the isoxaozline ring and 2-pyrrolidinone tail substituent destroys agonist activity.[11] Recognizing this, the designed structure in Figure 1 incorporates a methylene linker between the core and tail regions as a means to limit intrinsic agonism. Indeed, the PAM-agonist compounds 4-7 also incorporate a methylene linker in this region. In addition, the proposed structure in Figure 1 includes a benzene ring in the tail portion, a structural motif observed in Compounds 3-7. Structure-activity studies performed by Davoren et al. suggest that aromaticity of the pendant region affords the ability to pi stack with surrounding amino acids.[6] Literature Compounds 4-7 also include a heterocyclic ring off of the aromatic substituent. Specifically, the 4-oxazole structure utilized by Davoren et al. in Compound 6 was incorporated in the proposed isoxazoline.[7] Other attempts on the part of Davoren and colleagues to vary the heteroaryl tail contributed to increased clearance of the drug in the body and ultimately proved unsuccessful.

The proposed isoxazoline structure in Figure 1 also incorporates the use of a hydroxy substituted cyclohexyl group in the top portion as introduced by Compound 4 in Table 1. Indeed, use of a hydroxyl substituent is also seen within Compounds 5 and 6. In general, research suggests that the top region is able to tolerate a high degree of variability.[8] However, the amide linker between the top and core portions occupies an important structural motif as it contributes to an intramolecular hydrogen bond. In their analysis of the relationships governing structure binding at the allosteric site, Davoren et. al highlight the importance of the intramolecular hydrogen bound to lock the core into a position that allows for hydrogen bonding with surrounding amino acids.[6] Therefore, the proposed structure in Figure 1 retains the use of an amide linker, as demonstrated by Compounds 4 and 5.

Synthetic Route

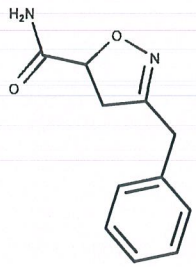


Figure 3: Simplified Isoxazoline Structure

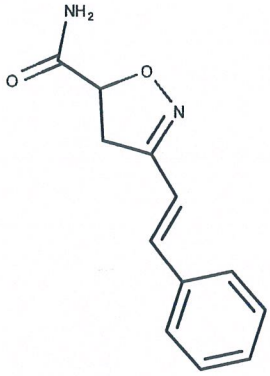


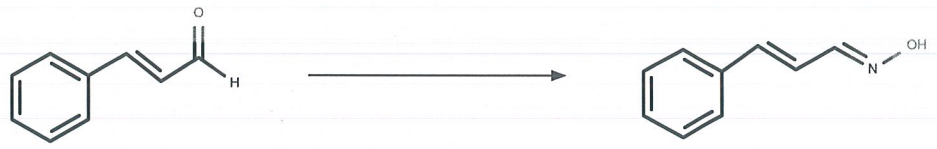
Figure 4: Adjusted isoxazoline structure targeted within the synthetic approach

To explore the synthesis of the proposed novel isoxazoline, a simplified version of the structure, illustrated in Figure 2, was targeted in the synthetic approach. Synthesis of a prototypical compound makes use of readily available reagents and reactants. It circumvents the need to expend money to purchase the chemical compounds necessary for target structure formation prior to any reassurance that the synthesis will succeed.

Through the design and synthesis of several hydroxamic acid derivates as potential antitrypanosomal agents, Rodrigues et al. demonstrate the use of a 1,3 dipolar cycloaddition in the formation of isoxazolines.[12] A 1,3 dipolar cycloaddition involves the reaction of a 1,3 dipolar compound with a dipolarophile, forming a five membered ring.[13] A 1,3 dipolar compound, like nitrile oxides, are electron rich charged dipoles that readily interact with a dipolarophile, an electron poor compound often containing electron withdrawing groups. In the cycloaddition, four electrons of the 1,3 dipole interact with two electrons of the dipolarophile to form a cyclic five membered structure, often containing heteroatoms like nitrogen or oxygen.

In the 1,3 dipolar cycloaddition, Rodrigues et al. utilized an alkene as the dipolarophile and through a series of reactions, transformed an aldehyde to a nitrile oxide as the 1,3 dipolar compound.[12] The proposed synthetic route applies the synthesis of Rodrigues and colleagues to the compound illustrated in Figure 3. However, the end synthetic isoxazoline structure was adapted to that illustrated in Figure 4 to make use of readily available starting reagents, cinnamaldehyde and acrylamide. Cinnamaldehyde was chosen as the starting aldehyde and converted to cinnamaldehyde oxime in Step 1 of Figure 5. In step 2, the cinnamaldehyde oxime is oxidized to a nitrile oxide, the 1,3 dipolar compound. Finally, the nitrile oxide reacts with the dipolarophile, acrylamide, to form the end isoxaozline product in Step 3.

Step 1: Formation of an oxime from the starting aldehyde

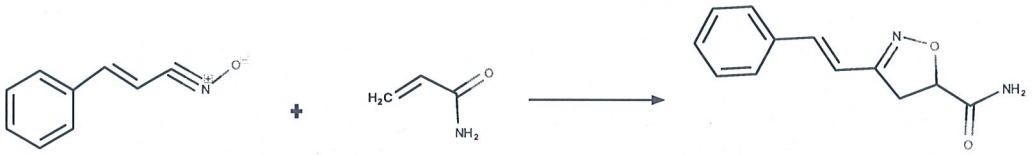


Cinnamaldehyde Oxime

Step 2: Conversion of an oxime to a nitrile oxide

Oxime Nitrile Oxide

Step 3: Reaction of the nitrile oxide with an alkene to form the isoxazoline



Nitrile Oxide Acrylamide Isoxazoline

Figure 5: Proposed synthetic route in the formation of the desired isoxazoline using cinnamaldehyde and acrylamide

Characterization of Reactants

Prior to attempting cinnamaldehyde oxime formation, the reagents were characterized using thin layer chromatography. In addition to a brown/orange liquid inside the reagent bottle of trans-cinnamaldehyde, a hardened crystal-like substance was also noted. This hardened substance was hypothesized to result from the oxidation of cinnamaldehyde. To confirm cinnamaldehyde was still present, the liquid substance was analyzed using IR spectroscopy. The IR spectrum displayed in Figure A.1 resembles that reported in literature for cinnamaldehyde, including a strong carbonyl stretch at 1674.95 cm^{-1} and relative peaks at 3061.08 and 3028.96 cm^{-1}, indicative of aromaticity. Therefore, IR provides confidence that cinnamaldehyde is present as the liquid substance within the bottle.

The chemical reactants of interest, cinnamaldehyde and acrylamide, were characterized using thin layer chromatography. In general, cinnamaldehyde appeared as an elongated streak with an average R_f value of 0.64. Meanwhile, acrylamide visualized as a single distinct spot with a R_f value of 0.47.

Discussion and Results

Below, Table 2 summarizes the results of three methods utilized in the formation of the cinnamaldehyde oxime. GC-MS analysis was carried out under a scan mode with an oven temperature beginning at 70°C ramping to 230°C at 20°C/min and finishing at a final temperature of 270°C. In addition, the inlet temperature was set at 250°C. Table 3 provides the details of analysis by TLC. All TLC was conducted on

aluminum-backed silica plates using a 90% ethyl acetate, 9% petroleum ether, and 1% acetic acid solvent system visualized with ultraviolet light.

Method	1	2	3
Reagents	- Cinnamaldehyde - Hydroxylamine hydrochloride	- Cinnamaldehyde - Hydroxylamine hydrochloride	- Cinnamaldehyde - Hydroxylamine hydrochloride - Sodium hydroxide
Variations in Method	Use of microwave heating to promote reaction	Reaction mixture stirred at room temperature for about 2 hours	Reactants were added to a 1:1:2 mixture of water, ethanol, and ice. The reaction mixture was stirred for about 18 hours
Product Description	Dark golden brown solution	Dark golden solution with precipitate particles	Golden brown solution
TLC Analysis	Spot elongated and moved closer to starting line.	Spot elongated and moved closer to starting line. However, TLC analysis following extraction produced notably different results.	Spot elongated and moved closer to starting line. However, TLC analysis following extraction demonstrated a more widened streak.
GC-MS Analysis	Not conducted	Cinnamaldehyde oxime presence was not noted. In addition, no higher mass compounds similar to that of cinnamaldehyde oxime were indicated.	74.526% cinnamaldehyde oxime noted at a running time of 13.093 minutes; corresponding to the maximum peak.
Percent Yield	----------	----------	159%

Table 2: Summary of three different synthetic methods explored in the formation of cinnamaldehyde oxime. For each method, the reagents and variations unique to the method are described, and the product produced characterized with a physical description, TLC, and GC-MS. Only notable GC-MS features are described; for the entire spectra, see the appendix.

Method	1	2	3
Initial	Large round spot; R_f = 0.72	Long spot; R_f = 0.72	One large round spot; R_f = 0.78 Smaller elongated spot; R_f = 0.66
93 minutes	----------	Elongated streak; R_f = 0.28	----------
Final	Elongated Streak; R_f = 0.34	Elongated streak; R_f = 0.39	Elongated streak; R_f = 0.49
Following extraction	----------	Large spot; R_f = 0.64	Elongated, widened streak; Rf = 0.74

Table 3: Summary of TLC analysis of the three different synthetic methods employed in the formation of cinnamaldehyde oxime. TLC analysis was carried out on a silica, aluminum backed plate using a 90%

ethyl acetate, 9% petroleum ether, and 1% acetic acid solvent system and visualized using ultraviolent light.

Method 1 was adapted from the work of Rodrigues et al, who microwaved their reaction mixture for thirty minutes. However, microwave heating in Method 1 was not allowed to progress for the total time of thirty minutes because of rapid boiling of the solution. The solution boiled almost immediately upon heating, quickly overflowing the Erlenmeyer flask and coating the microwave. Therefore, this synthetic method was not progressed for further analysis as the use of the microwave limited the extent of the reaction and produced noxious fumes.

Thin layer chromatography of Method 2 demonstrated that the central spot moved toward the starting line and elongated as the reaction progressed (Table 3). However, difficulties ensued when attempting to extract the desired compound from the mixture. As cinnamaldehyde was originally dissolved in ethanol, the ethanol content of the product mixture promoted miscibility of the aqueous and organic layers. A series of rotovapping finally afforded separation of the two layers. However, TLC analysis of the product following extraction was notably different than the TLC results immediately following the reaction. Indeed, the TLC spot following extraction more closely resembles that of the initial unreacted mixture. In addition, the final product also appeared to contain water droplets. Therefore, it is likely that the extraction process disrupted the identity of the product or the wrong extraction layer was chosen for isolation. Analysis by GC-MS confirmed that no cinnamaldehyde oxime was found in the product solution.

Of the three methods attempted in cinnamaldehyde oxime formation, Method 3 proved the most successful. TLC analysis demonstrated the elongation of the two distinct spots noted in the initial reaction mixture (Table 3). As expected with the synthesis of a more polar compound, the TLC spot following the reaction moved closer to the starting line. Analysis by GC-MS confirmed the presence of cinnamaldehyde oxime, emerging at a run time of 13.093 minutes (Figure A.___) and the disappearance of a peak at 11.544 min, noted in the GC-MS analysis of the reactant mixture (Figure ___). The success of Method 3 can be contributed in part to the inclusion of sodium hydroxide in the reaction mixture, as it ensures the neutralization of any hydrochloric acid produced in the reaction. Furthermore, use of diethyl ether to extract the desired product proved more successful than previous Method 2 attempts using methylene chloride. Altogether, reacting cinnamaldehyde with hydroxylamine hydrochloride in the presence of sodium hydroxide allowed for the successful formation of cinnamaldehyde oxime. However, a percent yield of 159% was noted, suggesting that other impurities are present and the cinnamaldehyde oxime has yet to be successfully isolated.

Method	4	5
Reagents	- product mixture produced using Method 2 of Table 2 - cyanuric chloride - trimethylamine - acrylamide	- product mixture produced using Method 2 of Table 2 - cyanuric chloride - trimethylamine - acrylamide

Variations in Method	No filtration of the mixture	Reaction mixture gravity filtered prior to addition of acrylamide and vacuum filtered following reaction completion
Product Description	An opaque off-white solution that separated into an off-white solid layer and a golden yellow liquid layer	A milky yellow opaque solid layer and a dark orange-brown liquid layer
TLC Analysis	Initial two spots merged and moved closer to starting line	Initial two spots merged to produce an elongated streak covering the distance the solvent traveled
GC-MS Analysis	No higher-mass compounds indicative of the isoxazoline noted	

Table 4: Summary of three different synthetic methods explored in the formation of the isoxazoline. For each method, the reagents and variations unique to the method are described, and the product produced characterized with a physical description, TLC, and GC-MS. Only notable GC-MS features are described, for the entire spectra, see the appendix.

Method	4	5
Initial	Small Spot 1; $R_f = 0.55$ Small Spot 2; $R_f = 0.70$	Small Spot 1; $R_f = 0.67$ Larger Spot 2; $R_f = 0.81$
Final	A thin small spot; $R_f = 0.18$	Elongated streak covering the distance the solvent traveled

Table 5: Summary of TLC analysis of the three different synthetic methods employed in the formation of the isoxazoline. TLC analysis was carried out on a silica, aluminum backed plate using a 90% ethyl acetate, 9% petroleum ether, and 1% acetic acid solvent system and visualized using ultraviolent light.

GC-MS analysis of the product produced using Method 4 suggests that no larger mass compounds, including the desired isoxazoline compound were produced. These results most likely stem from the use of the product produced by Method 2 as a reactant. Analysis of the Method 2 product mixture suggests that cinnamaldehyde oxime was not present, thereby depriving the Method 4 reaction mixture of the necessary starting reactants. In addition, no filtration was utilized in Method 4, thereby leaving unreacted acrylamide and isocyanuric acid in the product mixture. Isocyanuric acid is a solid byproduct produced following addition of cyanuric chloride and trimethylamine.[12]

In contrast to TLC analysis of Method 4, Method 5 resulted in an elongated streak. Indeed, there were several marked differences between the results of these synthetic methods. For example, addition of cyanuric chloride did not produce a change in temperature in Method 4, while it resulted in rapid boiling of the solution in Method 5. These differences could be due in part of the differences between the starting cinnamaldehyde oxime solutions. Only Method 5 started with a product mixture confirmed by GC-MS to contain cinnamaldehyde oxime.

GC-MS of the product mixture produced by Method 5 confirmed the milky yellow solid to be unreacted acrylamide (Figure A.4). Meanwhile, GC-MS analysis of the liquid layer suggests that higher mass compounds were produced. However, it does not provide definitive evidence of the isoxazoline. For example, Figure A.5 notes the emergence of higher mass compounds at 20.287 and 20.291 minutes. However, MS data suggests that these compounds have a total mass of about 279 g/mol, much higher

than that of our desired structure. In addition, whereas a base peak of 149 was noted, the structure of our desired compound does not provide an explanation for the identity of this peak. Interestingly, the largest MS peak, emerging at 12.801 minutes was found to be 2-propenenitrile. This results suggests that perhaps the cinnamaldehyde was not sufficiently oxidized, resulting in an abundance of 2-propenenitrile. Future experiments should attempt alternative methods in the oxidation of the oxime and continue to explore the reaction of the nitirile oxide with acrylamide to form the desired isoxazoline. Altogether, Method 5 does not provide proof of isoxazoline formation and additional analysis is needed.

Experimental Section

Formation of Cinnamaldehyde Oxime

Method 1: Initially, formation of the cinnamaldehyde oxime was attempted through Method 1 in Table 2 utilizing a microwave. To a 2.50 mL of cinnamaldehyde dissolved in 50 mL ethanol was added a hydroxylamine hydrochloride solution prepared by dissolving 4.1720 g in 10 mL of DI water. Initial addition of hydroxylamine hydrochloride to cinnamaldehyde produced an opaque yellow solution with a milky precipitate. After transferring the mixture to the microwave, heating resulted in the overflow of the reaction mixture and vigorous boiling. After about a minute, heating in the microwave was terminated as a result of the violent popping and bubbling in addition to pungent fumes.

Method 2: A solution of cinnamaldehyde was prepared by dissolving 2.52 mL of cinnamaldehyde, as measured by a 5 mL syringe, in 50 mL ethanol. In addition, 4.1672 g of hydroxylamine hydrochloride was dissolved in 10 mL of DI water and added to the solution of cinnamaldehyde. Initial combination of the solutions in a round bottom flask produced a light-yellow solution with a yellow precipitate. The reaction mixture was submerged in a water bath at a temperature of about 24°C and stirred continuously for a total of 2 hours, 6 minutes. Following the reaction, an opaque orange/yellow solution with a white precipitate is produced.

To extract the product, 10 mL of methylene chloride and 5 mL of DI water are added in a separatory funnel. However, even after adding an additional 10 mL of methylene chloride, no distinct layers are formed. To reduce the ethanol content of the mixture and promote separation, the product mixture was rotovapped and followed up with extraction using 10 mL methylene chloride. Distinct layers failed to form, even after attempting to salt out the aqueous layer with addition of two spoonsful of sodium chloride. Two additional rounds of rotovapping followed up by extraction with sodium chloride and methylene chloride failed to produce distinct layers. However, the last extraction attempt provided separate layers when 10 mL of methylene chloride were added without shaking the separatory funnel and stirring the mixture with a spatula. The lower organic layer was dried using anhydrous sodium sulfate and filtered to remove the drying agent.

Method 3: The previous synthetic method was adapted according to a procedure by Ramòn et al. Using a 5 mL syringe, 2.52 mL cinnamaldehyde were measured out and added to a mixture containing 5 mL DI water, 5 mL ethanol, and about 10 mL of ice in a round bottom flask. The reaction mixture was placed on an ice bath to ensure that the temperature stayed below 30° C. In addition, 1.3976 g of hydroxylamine hydrochloride was added to the mixture, creating an opaque orange/brown solution with a white precipitate. The solution was connected to a drying tube and stirred continuously at room temperature for a total of 17 hours, 47 minutes. Extraction of the product was achieved using about 15

mL of diethyl ether. Separation occurred, producing an upper semi-transparent golden brown organic layer and a lower dark brown orange opaque aqueous layer. The organic layer was removed and the aqueous layer acidified by dropwise addition of 6 M HCl to obtain a pH of 6 according to pH test strips. The aqueous layer was re-added to the separatory funnel and extracted twice using about 7 mL portions of diethyl ether. The organic layers were combined and dried with three spatula-full scoops of anhydrous magnesium sulfate. Finally, the product solution was rotovapped under vacuum but without submersion in a hot water bath. For analysis by GC-MS, several drops of the product were dissolved to 1 mL using ethanol.

Formation of the Isoxazoline

Method 4: The first method involved a synthetic continuation of the product produced by Method 2. The product produced by Method 2 was placed on an ice water bath and cooled to a temperature below 15°C. To the cooled product solution, 1.87 mL of trimethylamine, a transparent slightly yellow liquid, was added along with 1.842 g of cyanuric chloride, a fine white powder. Prior to addition these reagents, the product mixture was cooled to a temperature below 15°C using an ice water bath. After adding trimethylamine and cyanuric chloride, the reaction mixture was allowed to remain on the ice bath for 15 min. Afterwards, the ice bath was removed and the mixture stirred continuously while connected to a drying tube, for a total of thirty minutes. To the opaque milky grey solution, 4.26 g of acrylamide, a white crystalline solid, was added. No color change was observed upon addition of the acrylamide. The mixture was stirred for 24 hours to produce a thick opaque milky solid and a golden yellow/orange solution. Several drops of the liquid layer of the product mixture was dissolved in ethanol for analysis by GC-MS. TLC was utilized to monitor the reaction, providing analysis of the final product mixture and the reaction mixture prior to addition of cyanuric chloride and trimethylamine.

Method 5: The 6.272 g product solution produced using Method 3 in Table 2 was cooled to a temperature below 15°C. While the solution remained on the ice bath, 1.87 mL of trimethylamine was added via syringe along with 2.022 g of cyanuric chloride, a white power. Upon addition of cyanuric chloride, a gas was produced and the reaction mixture proceeded to boil rapidly even while on ice. The mixture was retained on the ice bath for 15 minutes, allowing the temperature to once again drop below 15°C. As a solid had formed, about 20 mL of diethyl ether were used to re-dissolve the mixture. The mixture was stirred at room temperature for 30 minutes while attached to a drying tube. By 30 minutes, a solid byproduct of peanut-butter color and consistency had formed within a dark golden brown solution. Using diethyl ether, the mixture was gravity filtered to remove the solid byproduct and returned to the round bottom flask. While placing the flask on an ice-water bath, 1.244 g of acrylamide, a white crystalline solid, was added. The ice bath was removed following solution and the mixture stirred at room temperature while attached to a drying tube for a total of 24 hours. At this time, a yellow-orange milky precipitate had formed along with a dark orange-brown liquid. To remove the precipitate, the mixture was vacuum filtered. A small portion of the solid product along and several drops of the liquid product were each dissolved in ethanol for analysis by GC-MS.

Conclusions

A novel isoxazoline structure containing cyclohexanol and oxazole substituents has been proposed as a potential PAM-agonist with potent PAM activity and weak intrinsic agonism. In addition, a synthetic route utilizing cinnamaldehyde and acrylamide and involving a 1,3 dipolar

cycloaddition has been introduced in the formation of an isoxazoline ring. The reaction of cinnamaldehyde with hydroxylamine hydrochloride in the presence of base provided a successful means to synthesize cinnamaldehyde oxime. To form the isoxazoline ring, cinnamaldehyde oxime was oxidized to a nitrile oxide and reacted with acrylamide. However, additional analysis is needed to confirm or disprove the presence of the desired isoxazoline. In addition, future research should explore alternative means to oxidize the oxime to the nitrile oxide, as a more successful route would promote the reaction with acrylamide.

After identifying a successful synthetic route, future work would extend the synthetic approach to the proposed isoxazoline structure. Future work would also include exploration of the compound's PAM ability at the M1 receptor in addition to pharmacokinetic and toxicological profiles.

Appendix

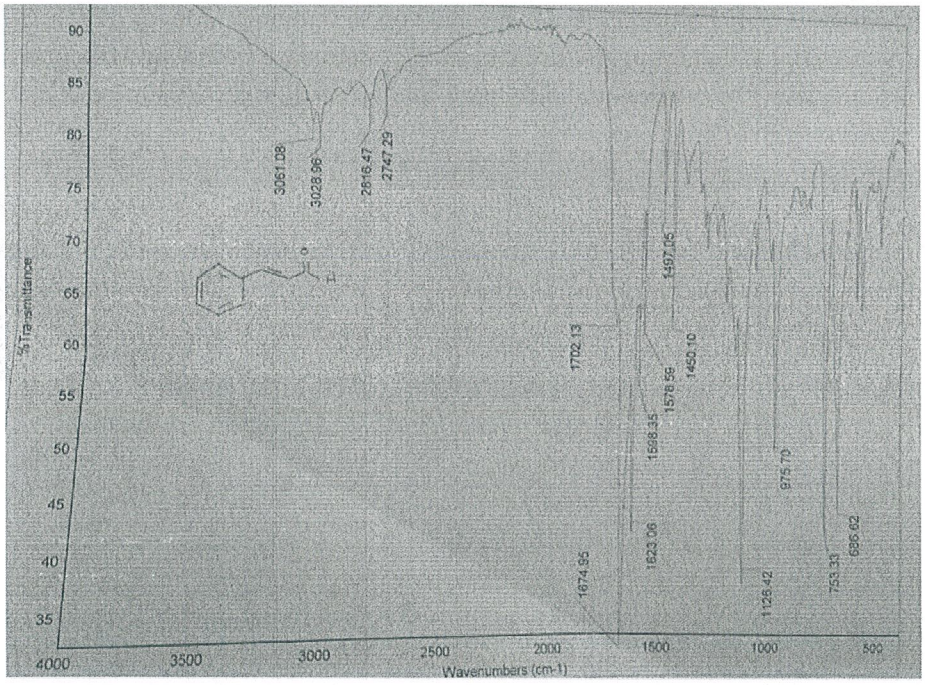


Figure A.1: Infrared spectroscopy of cinnamaldehyde reagent

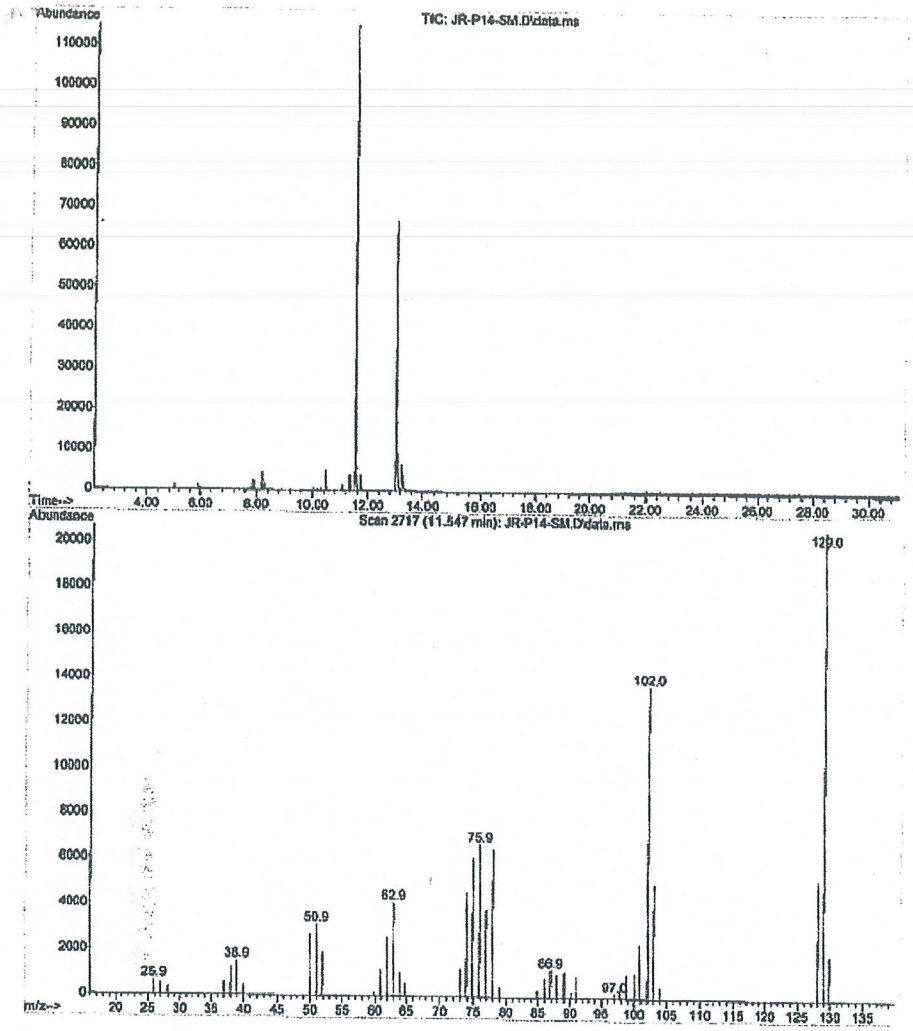


Figure A.2: GC-MS analysis of the starting reactant mixture of Method 3. MS data shown is for the GC peak emerging at 11.547 min. The second largest GC peak, emerging at a time of 13.044 min and representing a percent area of 33.52, was found to correspond to cinnamaldehyde oxime with a quality of 90.

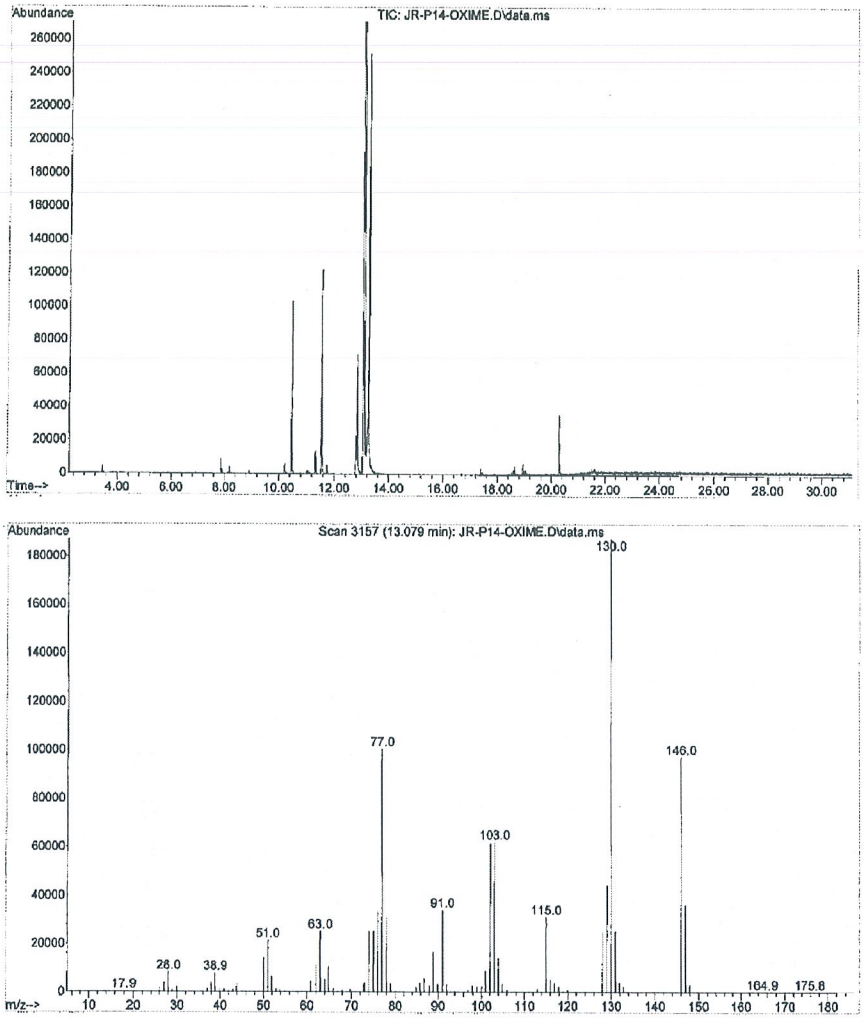


Figure A.3: GC-MS analysis following attempted formation of cinnamaldehyde oxime using Method 3. MS results are shown for the maximum GC peak emerging at 13.079 min and representing 74.526% of the total. On the basis of the MS data, the database identified the substance as cinnamaldehyde oxime with a quality of 70.

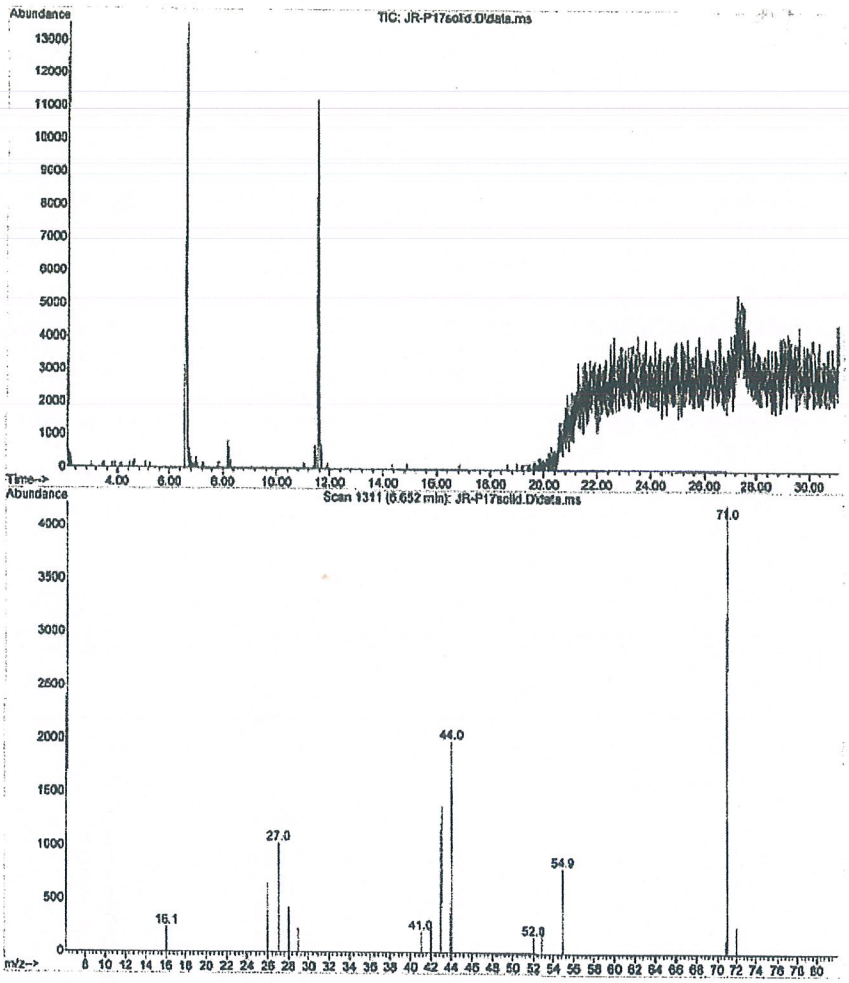


Figure A.4: GC-MS analysis of the solid product layer produced using Method 5 in the formation of the isoxazoline. MS data is shown for the GC peak emerging at 6.652 min, representing a 33.18% area and corresponding to acrylamide with a quality of 64.

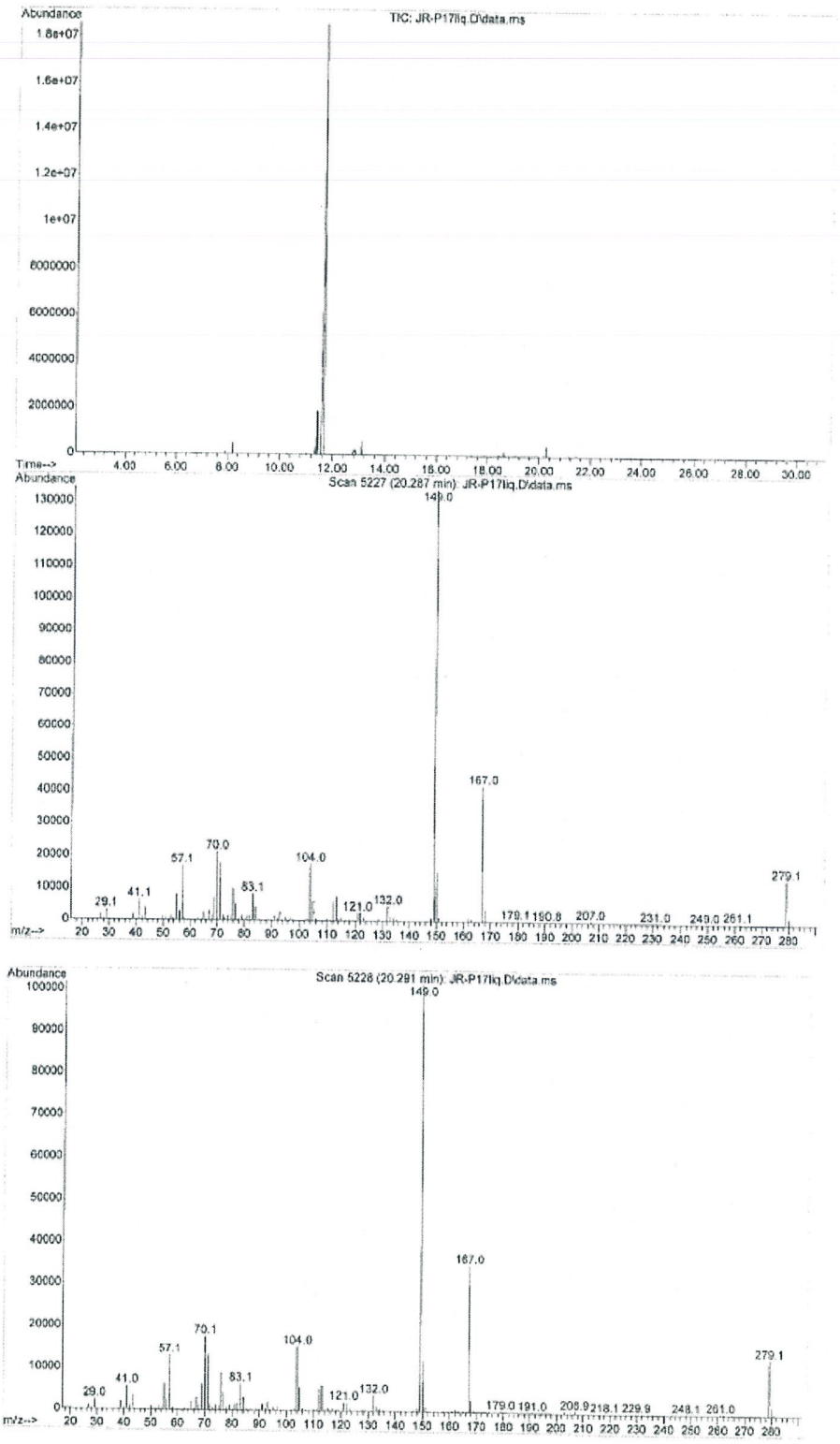


Figure A.5: GC-MS analysis of the liquid product layer produced using Method 5 in the formation of the isoxazoline. MS data is shown for the GC peaks emerging at 20.287 and 20.291 minutes, corresponding to substances of higher total mass.

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