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Synthesis of Triaminopyrimidine Derivatives for Inhibition of Inflammatory Caspases

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ABSTRACT Caspases are cysteine-dependent aspartic proteases whose functions are connected to different mechanisms of cell death and inflammation. As caspase-1 plays a significant role in the immune response, its activity has been of interest as a target for inhibitor development as its inhibition will reduce the levels of pro-inflammatory cytokines in inflammatory diseases, thus minimizing the symptoms that arise and serving as a possible therapeutic approach. Prior work discovered a family of potent inhibitors of caspase-1 with a common triaminopyrimidine scaffold. To further explore structure-activity relationship profiles of potential inhibitors of caspase-1, two different syntheses were attempted to create two new series of analogs for future activity studies. The syntheses presented here were conducted to investigate whether the variation of the amino groups on the pyrimidine scaffold effect the inhibition of caspase-1. The successful synthesis of the desired compounds has not yet been accomplished, but the optimizations of the reactions for further studies are reported.

INTRODUCTION

Caspases-1, 4, and 5 are cysteine-dependent, aspartate specific proteases that are involved in the inflammatory response. The activation of caspase-1 occurs upon the recognition of pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively) by NOD-like receptors. This leads to the generation of a multi-protein complex known as the inflammasome.[6] The biological substrates for caspase-1 are the inactive forms of pro-interleukin-1β and pro-interleukin-18, which are cleaved by caspase-1 to form the active cytokines IL-1β and IL-18.[5] Overactivation of caspase-1 has been linked to inflammatory cancers and autoimmune disorders, including type II diabetes, gout, and rheumatoid arthritis.[5] The involvement of caspases-1, 4, and 5 in the inflammatory and innate immune responses has led to the interest of designing and optimizing synthetic pathways that may yield potential inhibitors of caspase activity.

Research on inflammatory caspases has led to the discovery of a family of potent triaminopyrimidine inhibitors. The triaminopyrimidine scaffold was discovered through a library screening of potential scaffolds

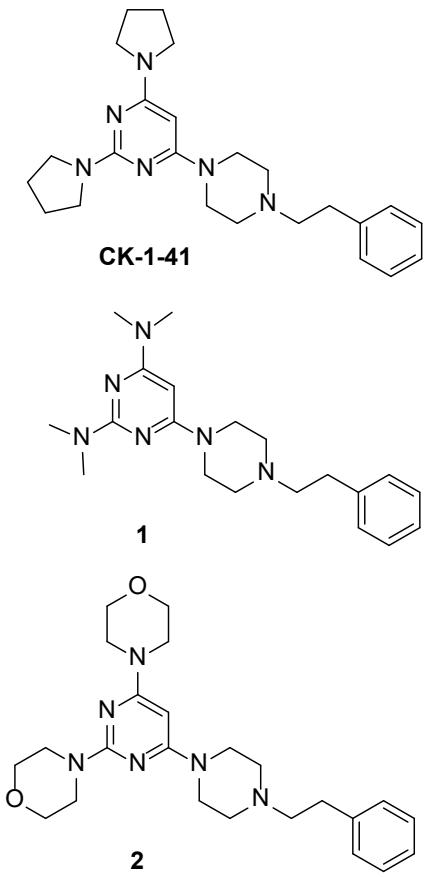

Figure 1. Structures of CK-1-41 and new analogs 1 and 2. CK-1-41 is the lead compound for development of new analogs.

for such inhibitors. Within the library screen, there were two lazaroids with a triaminopyrimidine heterocyclic scaffold that exhibited the highest potency in the inhibition of caspase-1.[2] With this in mind, further work was done to optimize the scaffold and aromatic linker length, which led to the successful synthesis of triaminopyrimidine derivatives that have also been screened as effective inhibitors of caspases-1, 4, and 5. The previously synthesized derivative, **CK-1-41**, (Figure 1) has been identified as the most potent inhibitor of caspases. Using a fluorescence-based activity assay, **CK-1-41** was determined to have an IC_{50} value of 56 ± 1 nM, which indicates that it is relatively potent in terms of inhibiting caspase-1.[3] The compound is equally effective in inhibiting caspases-4 and 5, but caspase-1 is the enzyme of most interest as it is the most characterized and understood of the human inflammatory caspases in terms of its functions and abilities.

The potency of triaminopyrimidine derivatives has shaped the focus of current research in the Karver lab at DePaul University. Previously discovered inhibitors of the inflammatory caspases have been identified to bind in either the active site of caspases or at an allosteric site.[3] However, compound **CK-1-41** and other triaminopyrimidine analogs synthesized by the Karver lab exhibited non-competitive inhibition, thus inhibitor binding prevents catalysis without altering affinity of the substrate for the active site. The exact binding location of the triaminopyrimidines is unknown, and its determination using activity-based probes and proteomic analysis is a focus in other projects in the Karver lab. The identity of the attached substituent on the piperazine ring has the ability to impact the mode of inhibition as well as the selectivity between inflammatory caspases.[3] Thus far, the ethylbenzene analog **CK-1-41** has demonstrated an increase in potency in the inhibition of the inflammatory caspases. Efforts are now focused on identifying and analyzing the effects of varying the amino groups on the pyrimidine scaffold. Initial structure-activity relationship studies will commence upon synthesis of ethylbenzene analogs of bis-dimethylamino pyrimidine (**1**) and dimorpholino (**2**) (Figure 1) for direct comparison to **CK-1-41**. Further variations can be done on the aryl ring using intermediates **4** and **6** (Scheme 1). To date, there have been limited reports of incorporating a piperazine ring onto a diaminopyrimidine core. Previous literature provided conditions for the synthesis of 4-(1-piperazinyl)-2,6-di-1-pyrrolidinylpyrimidine, which consists of refluxing the starting material with piperazine in pyridine.[1] This served as a starting point for the reaction trials presented here.

METHODS

General Methods

All chemical reagents utilized for the synthesis reactions were obtained from Enamine. Thin-layer chromatography (TLC) utilized silica gel 60 F254 coated aluminum foil sheets. TLC analysis was performed in 1:1 and 3:1 hexanes and ethyl acetate, as well as 95:5% dichloromethane (DCM) and methanol. All TLC solvent systems contained 1% triethylamine (TEA). Microwave reactions were conducted in a CEM Synthesizer Discover SP reactor system. Gas chromatography-mass spectrometry (GC-MS) was performed with the use of Agilent Technologies 7820A GC System and 5977E MS Detector. To retrieve the nuclear magnetic resonance ^{1}H (NMR) data, a Bruker 400MHz Neo Avance spectrometer was utilized. The solvent used for NMR samples was $CDCl_3$ and the reference peak was set for tetramethylsilane (TMS).

Benchtop Synthesis Reactions

All reactions completed on hot-plates and aluminum blocks followed a similar procedure. In either a 3-mL vial or 10-mL round-bottom flask, 0.100 mmol of the starting core (dimethylamino or dimorpholino core) was combined with 1 mL of the chosen solvent (THF or pyridine) and piperazine (equivalents of piperazine used in each reaction is shown in Table 1). A magnetic stir bar was added into the vial or round-bottom flask, which was then placed on a hot-plate for the time and temperature specified in Table 1. The reactions were monitored through TLC and GC-MS. Reactions were ended when no further progress was observed or if decomposition of the products was evident.

Microwave-Assisted Synthesis

The preparation of these reactions consisted of combining the starting material (dimorpholino or dimethylamino core) with excess equivalents of piperazine (Table 2) and 1 mL of the chosen solvent (if any solvent was used) in a microwave vial. The temperature and time of the reactions vary and can be found in Table 2. The vial containing the reagents was reacted in a microwave reactor. The reactions were monitored through TLC analysis to check the progress of product formation.

General Reaction Workup

Any reactions that exhibited potential product formation by TLC were subjected to an aqueous workup. Extraction for reactions that used tetrahydrofuran (THF) as the solvent proceeded by diluting the product with ~5 mL of ethyl acetate (EtOAc) followed by extraction with water (3 x 5 mL). For reactions utilizing pyridine as a solvent, the products were extracted with deionized water and DCM (2 x 5 mL). The aqueous phase was removed and the product was washed with sodium bicarbonate. Extraction for solvent-free reactions mirrored the extraction method of when pyridine and THF were used. All products were then dried with sodium sulfate anhydrous (Na_2SO_4) and solvent was removed in vacuo. Upon reaction completion, NMR samples were created by placing small amounts of the purified product into an NMR tube with $CDCl_3$ as the solvent.

RESULTS

Triaminopyrimidine derivatives of **CK-1-41**, specifically derivatives with varying amine substituents such as bis-dimethylamino and dimorpholino groups (**1** and **2**, respectively,

Scheme 1. Scheme for proposed syntheses of novel CK-1-41 analogs. The dimethylaminopyrimidine (**3**) and dimorpholinopyrimidine (**5**) have been reacted with piperazine (**7**) via nucleophilic aromatic substitution. Compounds **4** and **6** will undergo an S_N2 reaction with (2-bromoethyl)benzene to yield desired analogs **1** and **2**.

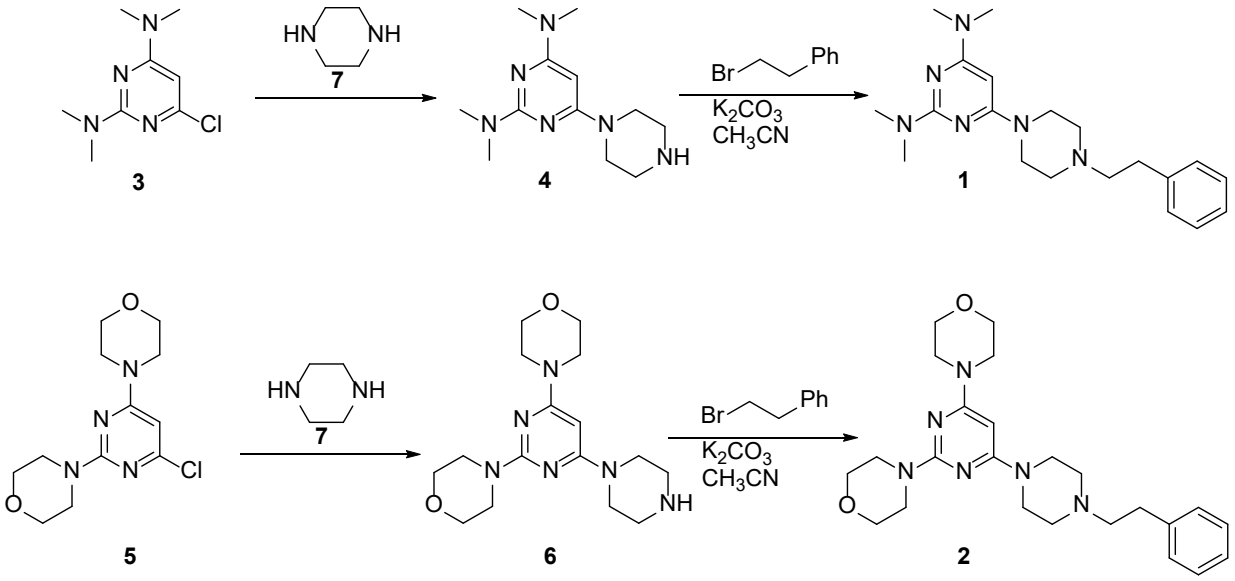

Figure 1) are novel compounds whose syntheses have yet to be accomplished in our lab or in others. They will be useful for providing information on what alkyl groups are tolerated for inhibition of caspase-1 at these positions, termed structure-activity relationships (SAR). The proposed general reaction schemes for the synthesis of these compounds are shown in Scheme 1. Once the piperazine is attached via nucleophilic aromatic substitution (S_NAr), the addition of an ethyl linked-aryl substituent can occur. These phenethyl analogs are mimics of the potent **CK-1-41** for a direct comparison of the effects of the amino group changes on caspase-1 inhibition.

The conditions attempted for the synthesis of the dimethylamino and dimorpholino variants via benchtop synthesis are shown below in Table 1. Four equivalents of piperazine (**7**) was insufficient to drive the substitution reaction to completion. The reaction was thus altered in terms of the equivalents of piperazine used. The equivalents of piperazine was increased to enhance the likelihood of the reaction going to completion. The alteration of the solvent from THF to pyridine did not improve the outcome as monitored by TLC. All of these reactions were unsuccessful as TLC analysis made it evident that the starting material (**3** or **5**) was present to a large extent in the samples. Although the reactions did not reach completion, the TLC plates did depict that some product may have been forming as there was an unknown immobile spot on the baseline of the TLC plate. Amines such as those in compounds **4** and **6** are highly polar and display very little mobility on a TLC. GC-MS analysis indicated that the reaction did not yield any identifiable product peaks. GC-MS was used in place of NMR analysis for all benchtop reactions due to the NMR being unavailable at the time. GC-MS is not an ideal method of characterization for the dimethylamino and dimorpholino compounds as their size and polarity make them difficult to ionize and therefore identify in the spectra.

Table 1. Reaction Conditions for the Benchtop Synthesis of Triaminopyrimidine Derivatives

Reaction Identity	Core[a]	Amount of core (mmol)	Equivalents of 7	Amount of 7 (mmol)	Solvent	Temp (°C)	Time (days)
MH-1-32	**3**	0.114	3.6 eq.	0.411	THF	45	1
MH-1-34		0.127	3.3 eq.	0.419	THF	50	6
MH-1-36		0.100	3.0 eq.	0.302	THF	50	7
MH-1-38		0.100	6.0 eq.	0.605	THF	50	2
MH-1-47		0.104	5.8 eq.	0.603	Pyridine	100	1
MH-1-48		0.104	5.8 eq.	0.603	Pyridine	100	6
MH-1-50		0.102	10 eq.	1.00	Pyridine	100	9
MH-1-33	**5**	0.100	3.9 eq.	0.389	THF	45	1
MH-1-35		0.100	3.8 eq.	0.376	THF	50	6
MH-1-37		0.100	4.2 eq.	0.423	THF	45	7
MH-1-39		0.100	6.3 eq.	0.625	THF	50	1
MH-1-49		0.198	3.5 eq.	0.700	Pyridine	100	6
MH-1-51		0.100	10 eq.	1.01	Pyridine	100	6

[a]For all reactions, 1 equiv. of core was used.

The electron-donating (EDG) amine groups make the attachment of a third nitrogen to the ring particularly difficult. The presence of the EDG groups makes the carbon atom undergoing substitution (asterisk in Figure 2) less electrophilic and thus less likely to react during nucleophilic aromatic substitution reactions (S_NAr). Throughout the optimizations completed thus far, it has been recognized that low equivalents of piperazine have not been effective enough to allow the reaction to go to completion. Benchtop reaction conditions require extensive amounts of reaction time and high temperatures. During these reactions, product formation could not be visualized on TLC plates in a timely manner, thus other methods were attempted.

In order to improve the yields of product, a new method, microwave-assisted synthesis, was implemented. Microwave energy has been found to be more time efficient in driving chemical reactions to completion.[4] Microwave reactions have also been shown to increase the percent yield of products as well as enable greener chemistry methods.[4] Microwave reaction conditions (Table 2) were initiated with smaller equivalents of piperazine and the use of a solvent (THF or pyridine). The use of a solvent in a microwave reaction can be useful if it is known to absorb microwave energy well. Solvents are split into three separate categories when analyzing their ability to absorb such energy: high, medium, and low. The solvents used throughout the syntheses in our work (THF and pyridine) fall into the low absorbance category.[6] This explains why the microwave conditions did not improve the outcomes in THF or pyridine. As the reactions progressed, it became evident that the conditions needed to be further optimized. Reactions were then carried out in excess

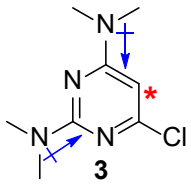

Figure 2. Electron donating groups disrupt nucleophilic aromatic substitution. The carbon atom that becomes less electrophilic due to the presence of the amine donating groups is depicted with an asterisk.

piperazine without the use of a solvent. Running reactions neat generally increases the rate of collisions between reactants and simplifies product isolation, especially in a microwave reactor. The reagents have the capability of absorbing the microwave energy, which allows superheating of the mixture that is typically not observed during conventional heating.[4] The analysis of the products formed through microwave heating with TLC revealed that some product formation occurred within a much more favorable time range (minutes vs. days), which indicates that microwave radiation and solvent-free reactions provided a kinetic advantage. The solvent-free reactions were done at a temperature of 146 ℃, which is the boiling point of piperazine. The specifics regarding equivalents, temperature, solvent choice, and duration are listed in Table 2.

The reactions that contained a solvent were unsuccessful as TLC analysis depicted starting material on the plate. This led to the recent optimization of carrying out the reactions in the absence of a solvent to allow more efficient collisions to occur at a high enough temperature

Table 2. Reaction Conditions for Microwave-assisted Synthesis of Triaminopyrimidine Variant

Reaction Identity	Core[a]	Amount core (mmol)	Equivalents of 7	Amount of 7 (mmol)	Solvent (1 mL)	Temp (℃)	Time (mins)	Crude Yield (%)
MH-1-54	3	0.104	2.9 eq.	0.308	THF	120	30	----
MH-1-56		0.104	10 eq.	1.01	THF	140	60	----
MH-1-62		0.108	10 eq.	1.02	THF	145	90	----
MH-1-64		0.105	10 eq.	1.07	pyridine	165	90	----
MH-1-65		0.106	10 eq.	1.01	----	146	30	----
MH-1-66		0.106	10 eq.	1.03	----	146	60	----
MH-1-68		0.100	10 eq.	1.00	----	146	90	----
MH-1-55	5	0.105	6.7 eq.	0.707	pyridine	140	60	----
MH-1-57		0.100	10 eq.	0.100	THF	140	60	----
MH-1-61		0.101	2.9 eq.	0.301	pyridine[b]	165	75	----
MH-1-69		0.100	10 eq.	1.03	----	146	65	86.2
MH-1-70		0.0997	10 eq.	1.09	----	146	90	>100

[a]For all reactions, 1 equiv. of core was used. [b]The volume of pyridine was increased to 4 mL.

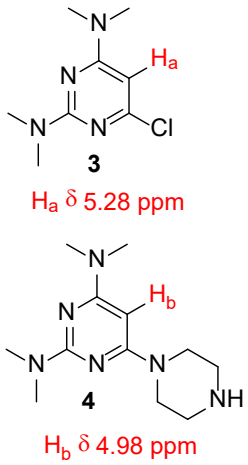

Figure 3. Proton of interest on triaminopyrimidine derivatives. As the electronegative chlorine atom is displaced by the substitution of the piperazine ring, the chemical shift moves upfield since the electron density around the nucleus slightly increases. Proton H_a is attached to the starting core, while H_b is the proton that indicates product formation.

(146 ℃). This optimization has shown that product is indeed forming and is on the right path of reaching completion. The reaction of MH-1-66 has shown the most progress thus far as its ^{1}H NMR spectrum (400MHz, $CDCl_3$) depicted peaks within the product region. As shown in Figure 3, the chemical shift of 4.98 ppm indicates product formation, while the peak at 5.28 ppm corresponds to the presence of starting material. The substitution of the chlorine atom by the piperazine ring at position 6 of the triaminopyrimidine core results in an upfield shift of the ortho proton on the NMR spectrum due to concept of electron density. The chlorine atom is an electron withdrawing group and thus pulls electron density away from the molecule, which decreases the overall electron density surrounding the nucleus. As the electron density around the nucleus decreases, there is a larger magnetic field felt by the nucleus and the signal is shifted downfield to a higher ppm. The attachment of the piperazine ring results in a shielded nucleus that now experiences a smaller magnetic field due to the piperazine increasing the overall electron density around the nucleus resulting in a ^{1}H signal that is shifted upfield to a lower ppm.

The ^{1}H NMR spectrum that depicts the prominent peaks from reaction MH-1-66 is shown in Figure 4. The reaction of MH-1-70 has also showed progress as the TLC plate depicted the presence of product as there is an immobile substance at the baseline and similar H_a and H_b peaks on the ^{1}H NMR spectrum. The percent yield of MH-1-70 was above 100%, which indicates that the sample is impure, most likely due to the presence of water and piperazine. Future samples may be subjected to high vacuum to thoroughly remove any residual solvent that is still present after rotary evaporation.

DISCUSSION

The microwave reactions have shown promise, producing small amounts of product, with starting material remaining. Future directions and optimizations of these reactions will be completed with the utilization of microwave-assisted synthesis, increasing the duration of the reactions, and maintaining 10 equivalents of piperazine in both the presence and absence of a solvent. It is likely that the temperature will be maintained at the boiling point of piperazine (146 ℃) for neat reactions. The idea of refluxing the reaction while in the microwave reactor may also be attempted in the near future. The work-up and analysis procedures will likely remain the same unless minor modifications are necessary. With a lack of research completed on the substitution of a secondary amine on the aromatic triaminopyrimidine core, the synthesis of these compounds is considered novel. However, future directions may also include researching possible catalysts or reagents that have the potential of favoring this nucleophilic substitution reaction to aid in the process of driving the reaction to completion. Once NMR analysis confirms the

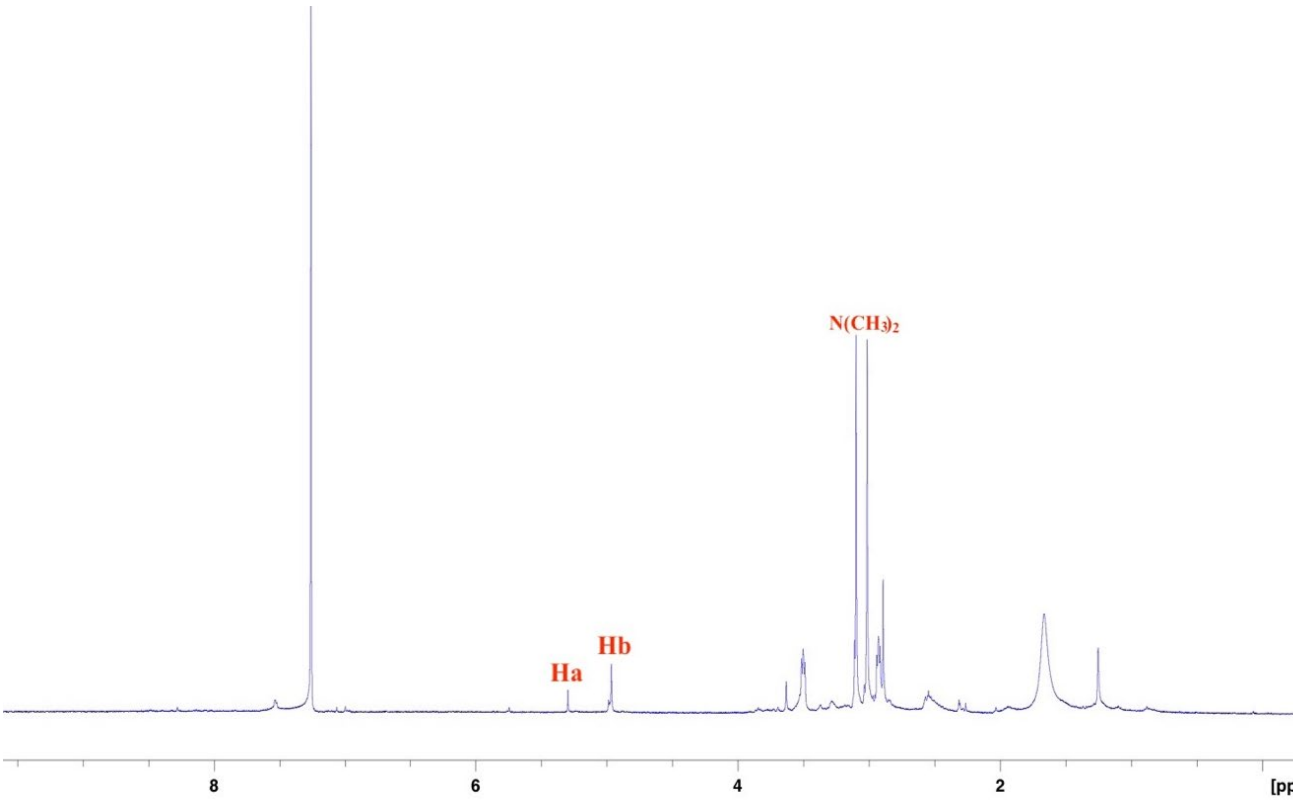

Figure 4. ^{1}H NMR Spectrum of MH-1-66. Proton Ha indicates the presence of starting material in the sample at a chemical shift of 5.28 ppm, while Hb depicts product formation at 4.98 ppm (refer to figure 4). The peaks ranging from 2.71-3.50 ppm refer to the methyl protons attached to the amine groups ($N(CH_3)_2$). Residual solvent peaks for $CDCl_3$ (7.30 ppm) and water (1.50 ppm) are depicted in the spectrum as well.

presence of significant quantities of product, it will be purified before proceeding with the nucleophilic substitution reaction with bromoethylbenzene, specifically the conversion of compound **4** to compound **1** and compound **6** to compound **2** (Scheme 1). The conditions for attachment of the ethyl benzene portion of the molecule are well established and should proceed without difficulty as seen throughout the synthesis of previous analogs within the Karver lab.[4] Once these compounds are synthesized and characterized, they will be screened for the inhibitory ability with inflammatory caspases. These compounds will provide future directions for new reaction schemes and optimizations as they will reveal patterns of the structure-activity relationship profile for triaminopyrimidines with these enzymes.

ACKNOWLEDGEMENTS

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