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Caroline McCormack April 3, 2023

Novel Quaternary Ammonium Compounds Derived from the Antimicrobial Natural Product Ianthelliformisamines A and C

by

Caroline McCormack

William Wuest, Ph.D.
Adviser

Chemistry

William Wuest, Ph.D.
Adviser

Julie Clennon, Ph.D.
Committee Member

Richard Himes, Ph.D.
Committee Member

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Caroline McCormack

William Wuest, Ph.D.

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Abstract

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Quaternary ammonium compounds (QACs) are a class of antimicrobials commonly used in hospital and household disinfectant and antiseptic products. During the COVID-19 pandemic, the usage of QACs has likely risen drastically due to increased cleaning and disinfection to slow the spread of SARS-CoV-2, leading to increased concentrations of QACs accumulating in the environment. This is concerning because bacteria can develop resistance to QACs if exposed to sub-inhibitory concentrations in the environment. QAC resistance is tied to the development and proliferation of resistance to clinical antibiotics, exacerbating the risk of antibiotic resistant infections. Combating the threats of QAC resistance and antibiotic resistance necessitates the development of novel QACs with increased efficacy against resistant pathogens and higher barriers to the development of resistance. Antibacterial natural products present opportune scaffolds for the development of novel QACs because of their diverse structures and the possibility for creating multicationic QACs and QACs with a dual mechanism of action. The aim of this project was to synthesize novel quaternary ammonium compounds derived from the marine natural product ianthelliformisamine C. Ianthelliformisamine C was synthesized in seven steps in 47% overall yield following a lengthy optimization of the final step, a challenging amide coupling with a difficult purification process. Preliminary results for the quaternization of ianthelliformisamine have been challenged by low reactivity, prompting changes in the synthetic route to the ultimate QAC analogs. Going forward, minimum inhibitory concentration and hemolysis assays will be performed on the QAC analogs to investigate their antibacterial activity and toxicity.

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1. Background: Quaternary Ammonium Compounds, COVID-19, and Antibiotic Resistance

1.1 Quaternary Ammonium Compounds

Quaternary ammonium compounds (QACs) are a class of disinfectants used widely in household and hospital settings. QACs are present in a variety of commercial products, including disinfectant sprays and wipes, mouthwash, hand sanitizer, and cosmetics, as well as industrial cleaning and water treatment applications.[1] These compounds are salts containing at least one quaternary, cationic nitrogen atom surrounded by four carbon-containing groups, where at least one substituent is a long hydrocarbon chain.[2] The amphiphilic nature of QACs allows them to interact with phospholipids in bacterial membranes. The nitrogen cation forms electrostatic interactions with the negatively charged phosphate heads of the phospholipids, and the long hydrophobic tails integrate into the hydrophobic center of the membrane. This disrupts the membrane, causing leakage of intracellular contents and disruption of ion gradients, leading to cell death.[3]

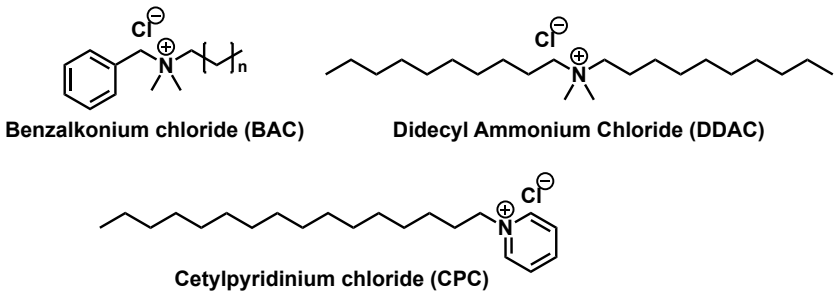
1.2 Disinfectant Usage During the SARS-Co-V-2 Pandemic

During the SARS-CoV-2 pandemic, the use of surface disinfectants likely increased as households, businesses, and institutions implemented cleaning and disinfection procedures to reduce the spread of SARS-CoV-2.[2] Of the 654 disinfectants listed on the Environmental Protection Agency's List N: Disinfectants for Coronavirus (COVID-19), 303 contain QACs as an active ingredient.[4] However, the increased use of QACs during the COVID-19 pandemic has environmental and public health implications because QACs accumulate in the environment due to their relatively long half-life.[2] Recent studies have demonstrated increased concentrations of QACs in human blood samples and residential indoor dust during the COVID-19 pandemic compared to before 2020.[5,6]

1.3 Antimicrobial Resistance

The increased use of QACs during the COVID-19 pandemic, and their resultant accumulation in the environment, is worrisome because of the development of QAC resistance.[2] Bacterial resistance to QACs has been well documented since at least 1946, and it occurs when bacteria are exposed to sub-inhibitory concentrations of QACs in the environment.[7, 8] While QAC resistance is concerning by itself because it renders commercially available disinfectants ineffective, it can also confer cross-resistance to clinical antibiotics.[2] This can occur through a single resistance mechanism, such as multi-drug efflux pumps capable of exporting both QACs and other antibiotics out of the cell, or through the acquisition of mobile genetic elements with multiple mechanisms of resistance to QACs and other antibiotics.[1, 9, 10] In this way, QAC resistance promotes the proliferation of antibiotic resistant bacteria, and increases the risk of antibiotic-resistant infections, which are responsible for 2.8 million infections and 35,000 deaths in the United States every year.[11]

1.4 Multicationic QACs



Scheme 1: Structures of some mono-cationic QACs commonly used in commercial disinfectants and antiseptic products.

To combat the threat of QAC resistance, it is necessary to develop novel QACs with improved efficacy against resistant pathogens. Commercially available QAC disinfectants, such as benzalkonium chloride compounds (BAC) and dialkyldimethyl ammonium compounds (DDAC), are monocationic, containing only one nitrogen cation (**Scheme 1**).[7] Previous research

has shown that multicationic QACs can remain effective against bacteria containing resistance genes for monocationic QACs,[12] and multicationic QACs exhibit a higher barrier to the development of resistance than monocationic QACs for Methicillin Resistant *Staphylococcus aureus* (MRSA).[7] Thus, multicationic QACs represent a promising direction for the development of next-generation antimicrobials with decreased chances of resistance development.

2. Ianthelliformisamine A and C and Antibacterial Natural Product-Derived QACs

2.1 Rationale for Exploring Natural Product-Derived QACs

We sought to leverage antimicrobial natural products to generate novel QAC scaffolds and expand the functionality of QAC antimicrobials. Previous work in our lab explored the synthesis of QACs derived from polyamines such as norspermidine.[13] These QACs were designed to mimic the structure of simple natural antimicrobial peptides with potent activity against biofilms. While QACs are typically only effective against planktonic bacteria, several of the polyamine-derived QACs exhibited biofilm eradication activity, demonstrating expanded functionality for QACs designed with antimicrobial natural products in mind.

In a continuation of this project, the synthesis of QACs derived from the easily accessible natural products quinine and nicotine was reported, demonstrating the feasibility of creating potent QACs derived from natural products.[14] A logical next step in this direction is to create QACs derived from antimicrobial natural products because they have the potential to retain the mechanism of action of the original natural product. This would yield an antimicrobial with a dual mechanism of action that may raise the barrier to the development of resistance. The beneficial effect of combining QACs and antimicrobial natural products has been shown by Okano et al., who appended a quaternary ammonium moiety to vancomycin and observed enhanced antimicrobial activity.[15] Additionally, quaternizing the amines of natural products with multiple

amines would generate multicationic QACs with novel structural features and the potential for enhanced efficacy against resistant pathogens.

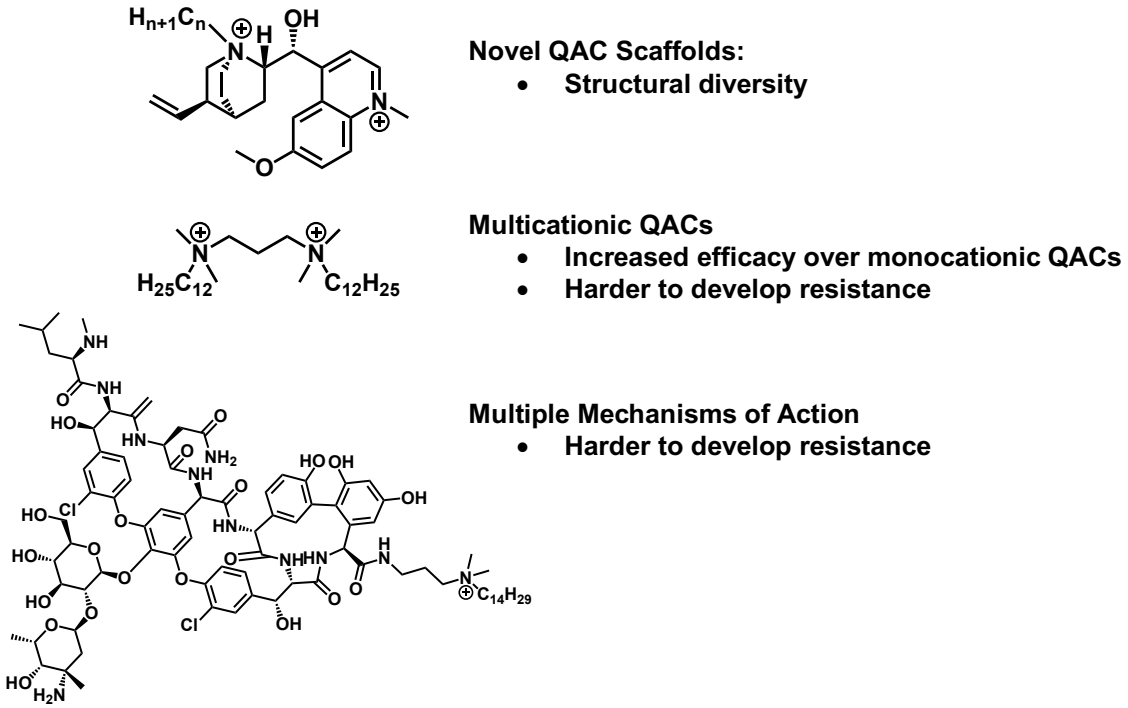
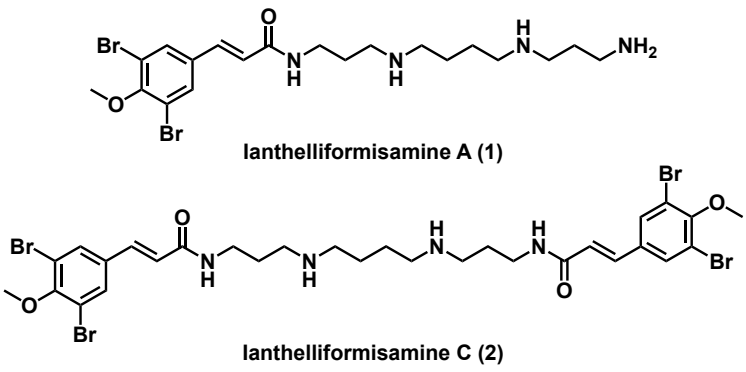


Figure 1. Rationale for exploiting antibacterial natural products as scaffolds for novel QACs. (Top): QAC derived from quinine synthesized by Joyce et al. (Middle): An example of a multicationic QAC synthesized by Foreman et al. (Bottom): Okado et al. appended a QAC moiety to the antibiotic vancomycin, resulting in enhanced antibacterial activity.

2.2 Ianthelliformisamine A and C: Bioactivity and Previous Work



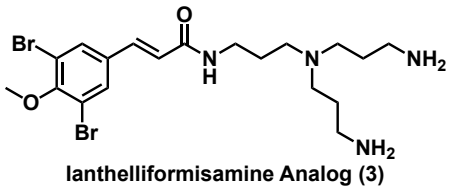
Scheme 2. Structures of marine natural products ianthelliformisamine A and C (**1** and **2**).

The natural products ianthelliformisamine A and C (**1** and **2**) were selected as candidates for novel QACs due to their inherent antimicrobial activity and multi-amine structure (**Scheme 2**). **1** and **2** are bromotyrosine-derivative antimicrobial natural products first isolated from the marine sponge *Suberea ianthelliformis* by Xu et al. in 2012.[16] In

minimum inhibitory concentration (MIC) assays performed by Xu et al., **1** exhibited selective activity against *Pseudomonas aeruginosa* (MIC 35 μM) and **2** demonstrated activity against *P. aeruginosa* (MIC 17.5 μM) and *S. aureus* (MIC 8.75 μM). **1** and **2** were first synthesized by Pieri et al. in 2014, who performed further investigations into the compounds' bioactivity.[17] They reported activity against *P. aeruginosa* PAO1 for **1** and broad-spectrum activity for **2** against *P. aeruginosa* PAO1, *S. aureus* DSM 799*, Klebsiella pneumoniae* KPC2 ST285*,* and *Enterobacter aerogenes* 289 (Table 1). The differences in the MIC values reported by Xu et. al. and Pieri et al. may be explained by a difference in methodology. Pieri et al. grew the bacterial strains in Meuller Hinton broth, while Xu et al. used cation-adjusted Meuller Hinton broth.[16, 17] Pieri et al. also investigated the potential for **1** and **2** to be used synergistically with other antibiotics to inhibit gram-negative pathogens, and they observed markedly lower MIC values when bacteria were treated with **1** or **2** in combination with 2 μg/mL of doxycycline (Table 1).[17]

	Minimum Inhibitory Concentration (μM)			
	S. aureus DSM 799	*E. aerogenes* 289	*P. aeruginosa* PAO1	*K. penumoniae* KPC2 ST285
Ianthelliformisamine A **(1)**	>200	>200	200	>200
Ianthelliformisamine C **(2)**	8.75	100	25	12.5
Doxycycline	56	<3	112	-
Ianthelliformisamine A **(1)** + 2 μg/mL doxycycline	-	>100	12.5	>100
Ianthelliformisamine C **(2)** + 2 μg/mL doxycycline	-	12.5	3.12	12.5

Table 1. Antibacterial activity and synergy with doxycycline of ianthelliformisamine A and C **(1** and **2)** as reported by Pieri et al.



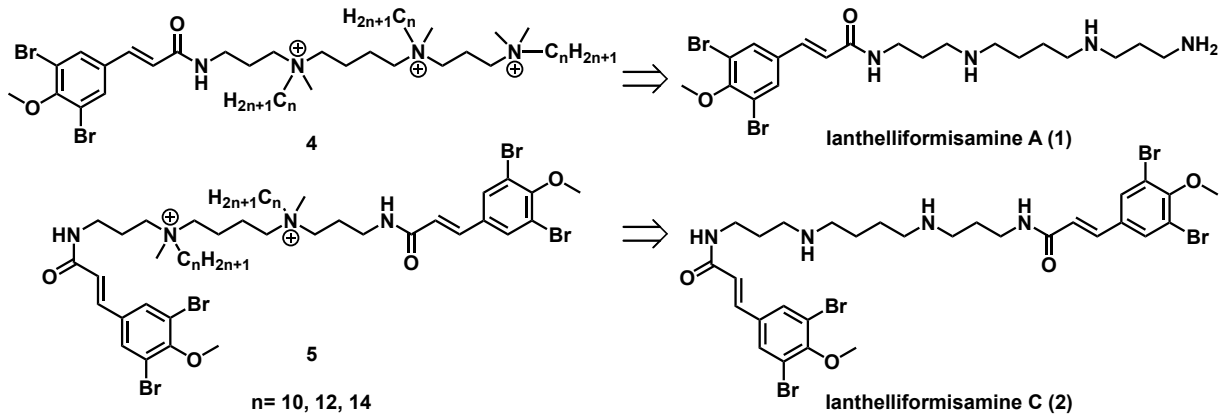
Scheme 3. Ianthelliformisamine analog synthesized and used in mechanistic studies by Pieri et al.

In initial investigations into the mechanism of action of an ianthelliformisamines analog **(3, Scheme 3)**, Pieri et al. demonstrated membrane depolarization but no membrane permeabilization after treatment with **3**. This indicates the ianthelliformisamine natural products may be inhibiting proton-coupled efflux pumps through disruption of the proton gradient.[17] By acting as protonophores, they may be depolarizing the membrane and rendering efflux pumps inactive. The amphiphilic character of the ianthelliformisamines may allow them to pass through the membrane and the basic amines may allow them to transport protons, resulting in membrane depolarization and cell death.

Preliminary cytotoxicity studies on **2** demonstrated no harmful effects on Chinese hamster ovary cells and human fibroblasts (IC_{50} = 1665 μM and 129 μM, respectively). It is worth noting that **1** and **2** were recently shown to inhibit several human carbonic anhydrases, key players in tumor pathogenesis and potential targets for novel anticancer drugs, with K_i values ranging from 0.20 to 9.08 μM.[18] **1** and **2** have also demonstrated activity against the malaria-causing parasite *Plasmodium falciparum*.[19]

2.3 Analog Design for Ianthelliformisamine-derived QACs

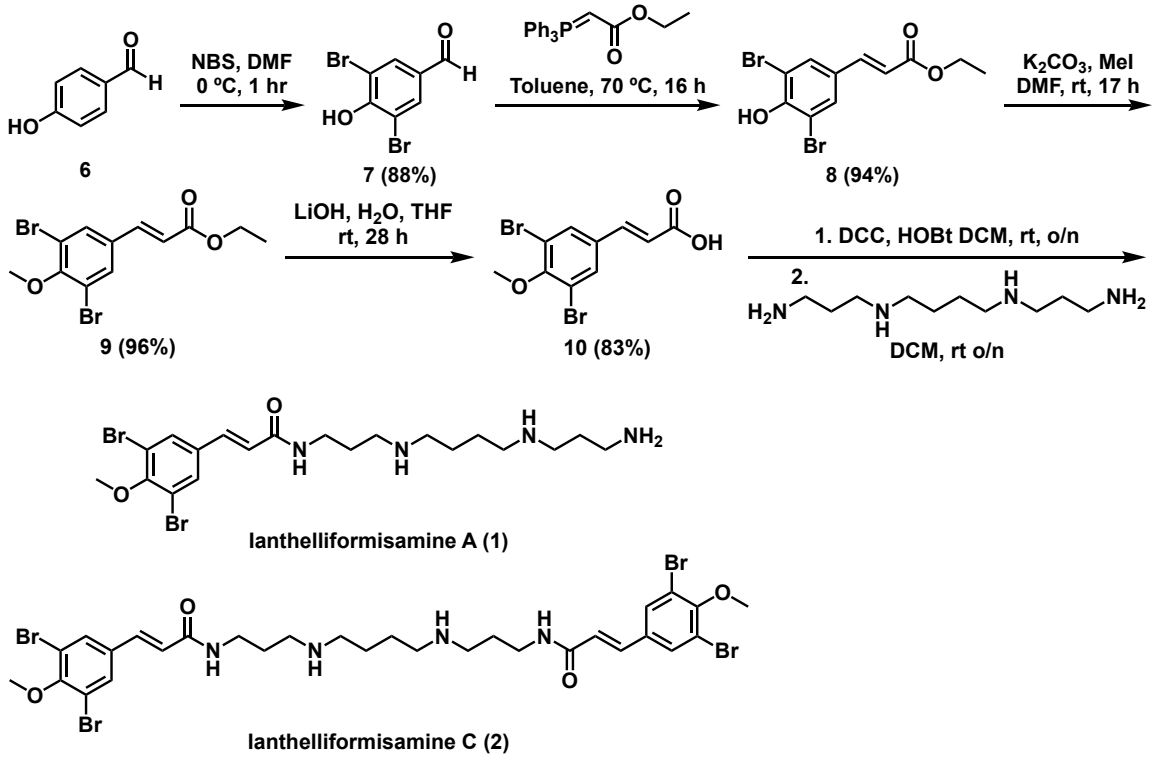
This project envisioned generating a set of six novel QACs from ianthelliformisamine A **(1)** and ianthelliformisamine C **(2)** (**Scheme 4**). Previous computational studies have demonstrated that the optimal carbon chain length for QACs is typically approximately 12 carbons.[20] For this reason, we aimed to make three QAC analogs for each scaffold with alkyl chains of 10, 12, and 14 carbons. We expect to see optimal activity with the 12-carbon analogs and aim to synthesize the the 10 and 14 carbon analogs for comparison.



Scheme 4. Analog design for a library of novel QACs derived from ianthelliformisamines A and C.

3. Synthesis of Ianthelliformisamine C

3.1 Synthesis of Ester and Carboxylic Acid Precursors to Ianthelliformisamines A and C

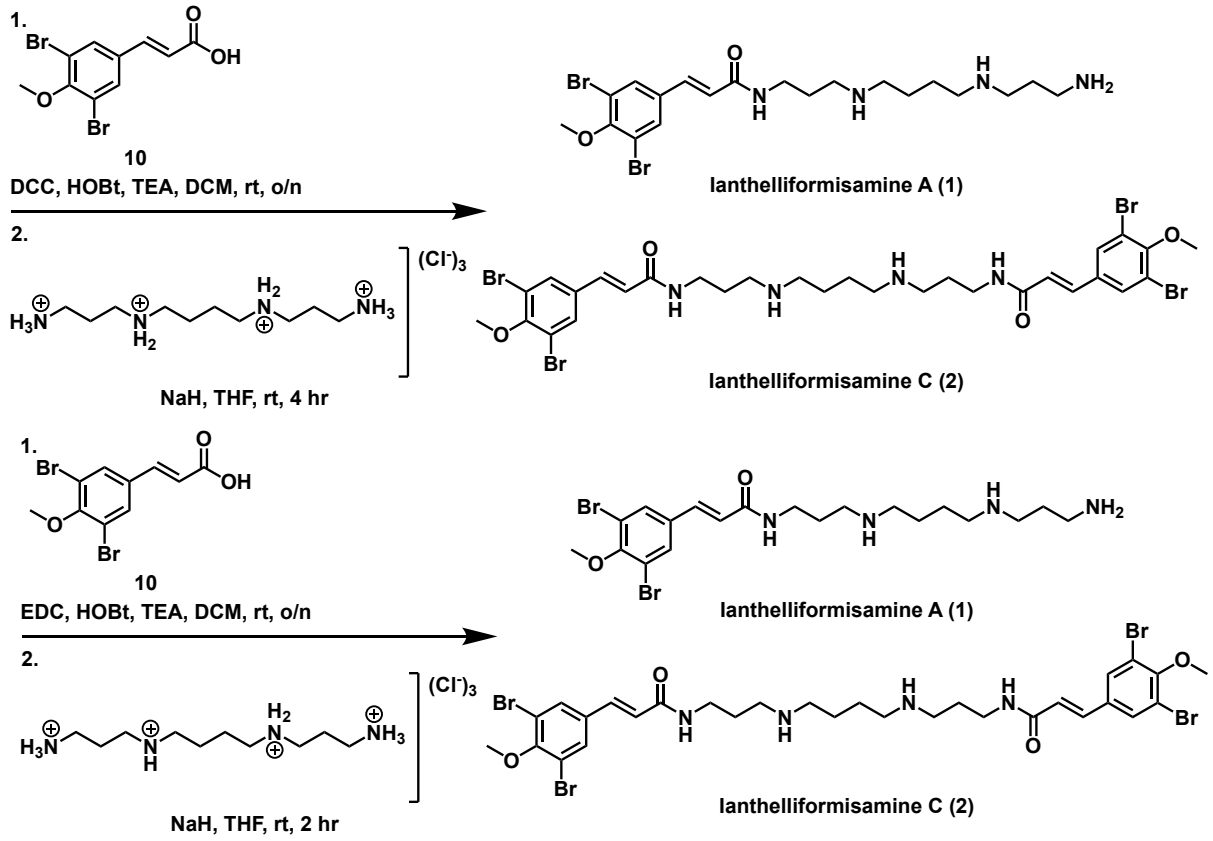


Scheme 5. Synthesis of ianthelliformisamine A and C (**1** and **2**).

3,5-dibromo-4-hydroxybenzaldehyde **(7)** was synthesized through a dibromination of 4-hydroxybenzaldehyde **(6)** in 88% yield. **8** was synthesized through a Wittig reaction between **7**

and carbethoxymethylene triphenylphosphorane. The phenol was then methyl protected **(9)**, and the ethyl ester was hydrolyzed to give the carboxylic acid **(10),** in 67% overall yield.

3.2 DCC and EDC Coupling Optimization

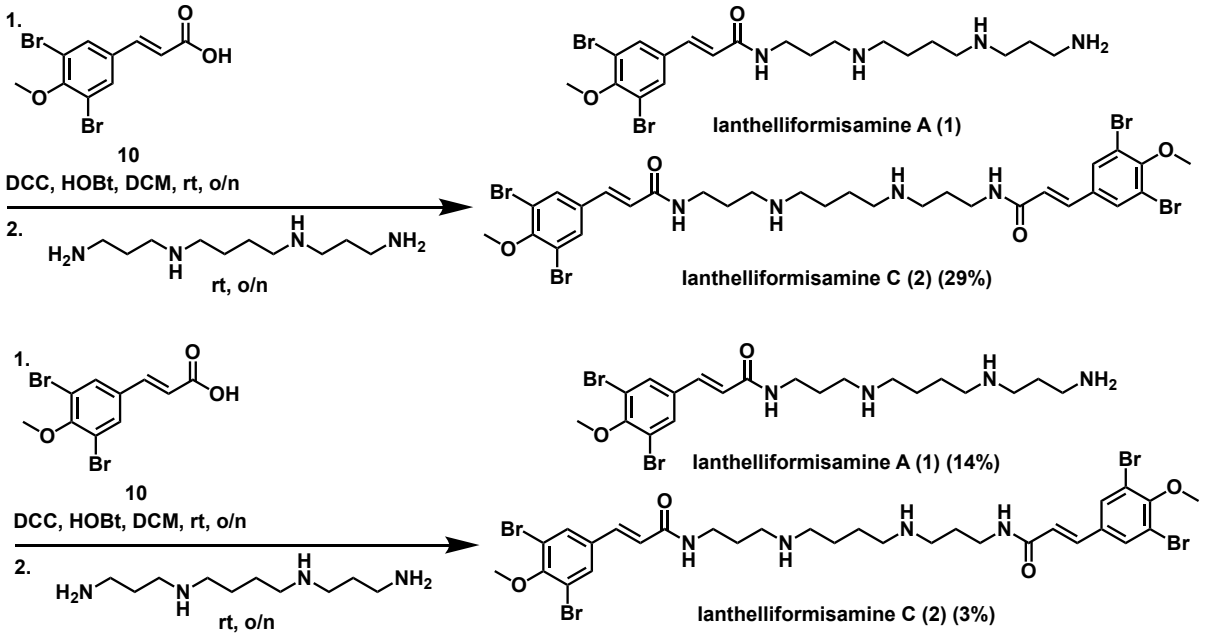


Scheme 6. (Top) DCC coupling between 3-(3,5-dibromo-4-methoxyphenyl)acrylic acid **(10)** and spermine hydrochloride salt to synthesize ianthelliformisamine A and C **(1** and **2)**. (Bottom) EDC coupling between **10** and spermine hydrochloride salt.

For the final step of the synthesis, Pieri et al. performed an amide coupling with N-N'-dicyclohexylcarbodiimide (DCC) and hydroxybenxotriazole (HOBt) to generate both **1** and **2** in the same reaction in a 3:7 ratio. When we performed the DCC coupling, we were not able to reproduce these results (**Scheme 6**). In our early trials of the DCC couplings, we were unable to obtain spermine and used the spermine hydrochloride salt in our reactions. The spermine hydrochloride salt was deprotonated with 3.9 equivalents of sodium hydride in tetrahydrofuran

(THF). After stirring for one hour, the mixture was filtered to remove the salt, and the filtrate was added to the coupling reaction. While it would have been ideal to use multiple equivalents of base to ensure full deprotonation, then perform an aqueous extraction to purify the spermine from the salt and remaining base, this was difficult because spermine is highly soluble in water. As a result, these reactions may have been hindered because of some of the spermine hydrochloride salt remained fully or partially protonated and thus less nucleophilic. We attempted using another coupling reagent,1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), with HOBt, **10,** and the spermine hydrochloride salt, but the formation of **1** and **2** was not observed.

Once we obtained spermine, we were consistently able to produce both ianthelliformisamine A and C through the DCC coupling, as confirmed by crude nuclear magnetic resonance (NMR) spectroscopy. However, the purification through column chromatography was difficult, and we were only able to isolate the natural products in complete purity when the reaction was run on a larger scale (400-600 mg). In one trial, we obtained **2** in 29% yield, and in another



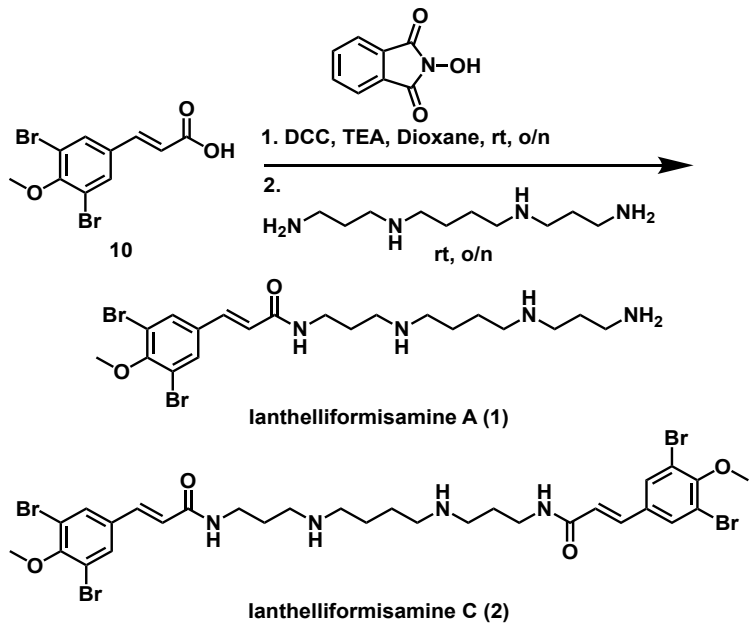
Scheme 7. DCC coupling between 3-(3,5-dibromo-4-methoxyphenyl)acrylic acid **(10)** and spermine to synthesize Ianthelliformisamine A and C **(1** and **2)**.

trial, we obtained a mixture of **1** and **2** in 14% and 3% yield, respectively (**Scheme 7**). The conditions of these reactions were very similar, and the difference in yields are primarily due to difficulties in purification since three successive column chromatography purifications were needed in order to obtain fully pure product.

The purification of this reaction was difficult due to the high number of species remaining in the mixture. Because the reaction does not go to completion, there were up to or more than eight species, including unreacted **10**, DCC, HOBt, and spermine, remaining DCC-coupled and HOBt-coupled activated esters, possible degradation or Michael addition products, and the two ianthelliformisamine products. This plethora of chemical species led to difficulties in purification, as the spots overlapped or co-eluted during column chromatography. The ionizable nature of the carboxylic acid **(10)** and the amines of **1** and **2** led them to stick strongly to silica and show inconsistent elution times. The polarity of the ianthelliformisamine products necessitated using very polar solvent conditions for column chromatography—mixtures of 30% methanol or isopropanol and 0.1-10% ammonium hydroxide in dichloromethane (DCM). Although inadequate, these column solvents were the most successful conditions for the purification of **2**. **1** or **2** were not observed in the fractions collected from either a normal-phase column with ethyl acetate and hexanes or a reverse-phase column with water, isopropanol, and ammonium hydroxide. It is worth noting that an aqueous work up is difficult for this reaction because the ianthelliformisamine products are soluble in both water and organic solvents like ethyl acetate. To fully remove them from the aqueous layer, it requires many repeated extractions with 10% MeOH in DCM.

The purification challenges led us to re-evaluate the DCC and EDC coupling procedures to increase the yields of **1** and **2** and simplify the purification. Recognizing that the low yields may be caused by incomplete consumption of the EDC- and HOBt-coupled activated esters, we let the

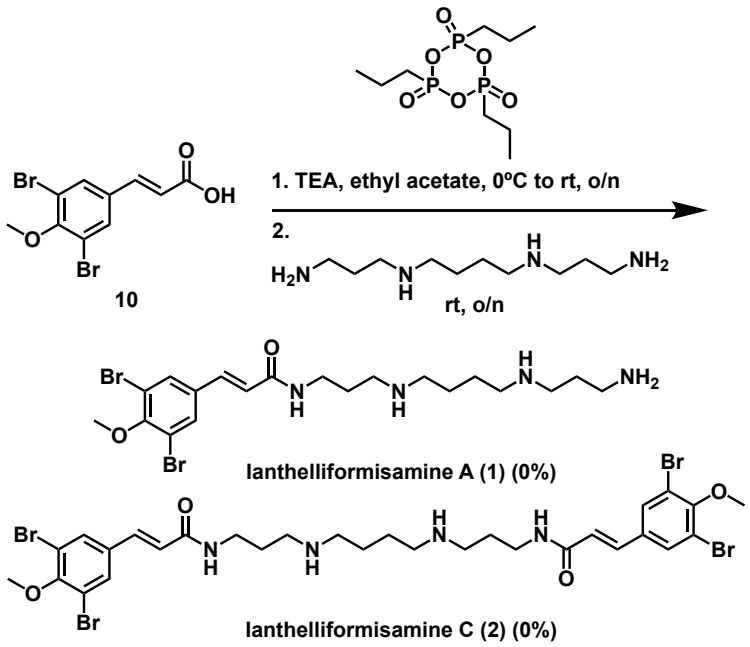
EDC coupling reaction run longer in attempts to drive it to completion. When the reaction ran for 17 hours, there were seven spots through thin-layer chromatography, and when the reaction ran for 43 hours, we observed six spots. While this may reflect further consumption of the starting material or EDC- and HOBt-coupled activated ester, it ultimately did not make a major difference in the purification process. When the temperature of the DCC coupling was increased to reflux in DCM overnight, only the HOBt-coupled and DCC-coupled activated esters were observed.



Scheme 8. DCC coupling with hydroxyphlamide, **10**, and spermine to synthesize **1** and **2**.

One factor that may have inhibited the DCC and EDC couplings was HOBt and interference from water. HOBt monohydrate was used for these reactions, resulting in the introduction of one equivalent of water per equivalent of HOBt to the amide coupling. We reasoned the water from the HOBt could be interfering with the reaction by hydrolyzing the DCC-, EDC-, or HOBt-coupled activated esters, thus lowering the yield of the reaction. When a combination of hydroxyphthalimide and triethylamine (TEA) was used as the base for the DCC coupling instead of HOBt, we did not obtain any of the carboxylic acid **(10)** at the end of the reaction, and we saw the formation of both **1** and **2** in the NMRs of the crude material (**Scheme 8**). However, we were unable to separate **1** and **2** from each other to report a yield.

Perhaps the most important factor impacting the amide coupling reactions is the electronics of **10**. The conjugated aryl and α-β unsaturated ketone system makes **10**, and its DCC-, EDC-, or HOBt-coupled activated esters more stable than their unconjugated counterparts, which may render it a poor electrophile and disfavor coupling with spermine. This would lead to low yields of **1** and **2** and significant amounts of leftover activated esters.



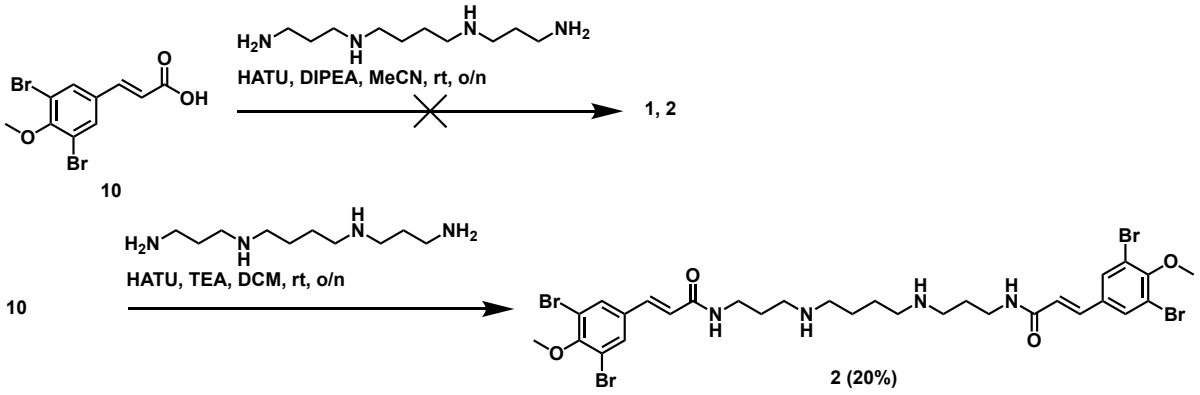
Scheme 9. The amide coupling with T3P, **10**, and spermine did not produce **1** or **2**.

3.3 Exploration of Alternative Coupling Reagents

Beyond DCC and EDC, other coupling reagents were explored in attempts to create a more electrophilic intermediate to couple with the spermine. When propylphosphonic anhydride (T3P) was used at the coupling reagent, **1** and **2** were not observed in the products (**Scheme 9**). After the spermine was added and the reaction was stirred at room temperature overnight, no change was observed through thin layer chromatography. There was still no conversion of the starting materials when the reaction was stirred at 45 ºC for eight hours and at 65 ºC for another two days.

We encountered limited success when hexafluorophosphate azabenzotriazole tetramethyl uronium (HATU) was used as the coupling reagent (**Scheme 10**). No product formation was observed when the reaction was performed with diisopropylethylamine (DIPEA) and acetonitrile

(MeCN). However, when the reaction was performed with TEA in dichloromethane, we isolated **2** in 20% yield.

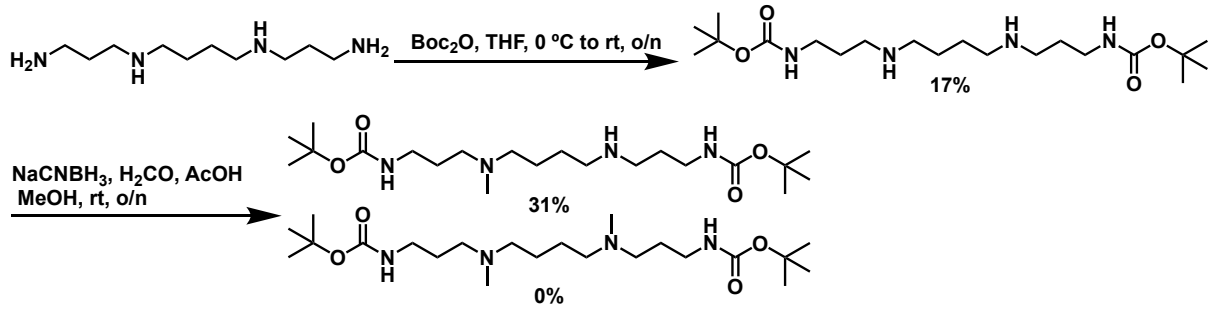


Scheme 10. Synthesis of **2** through an amide coupling with HATU.

3.4 Exploration of Protected Spermine Derivatives to Improve Amide Coupling

Despite achieving marginal success through the HATU coupling and DCC coupling with hydroxyphthalimide, low yields and purification difficulties still plagued the peptide couplings with **10** and spermine, leading us to consider alternate routes to our desired products. Khan et al. reported the synthesis of **1** and **2** through an EDC coupling with a protected derivative of spermine.[21] Khan et al. added tert-butoxycarbonyl (Boc) groups to the internal amines of spermine, which would reduce the hydrophilicity of the spermine and the resultant coupling products, likely making the purification process easier.[21] We envisioned that methylating the internal amines of spermine prior to the amide coupling would act similarly to the Boc-protecting groups to improve the purification by forming the less hydrophilic methylated ianthelliformisamine derivatives. The methylation would also remove one subsequent step to form the final QACs. We attempted to obtain dimethylated spermine through protecting the external amines with Boc groups, then methylating the internal amines through a reductive amination (**Scheme 11**). The di-Boc-protected

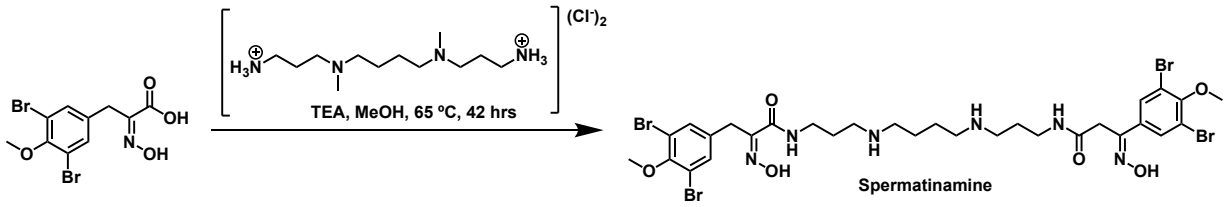
spermine was obtained in 17% yield. However, a subsequent reductive amination only afforded the mono-methylated product in 31% yield.



Scheme 11. Procedure for the Boc-protection and subsequent reductive amination of spermine to methylate the internal amines.

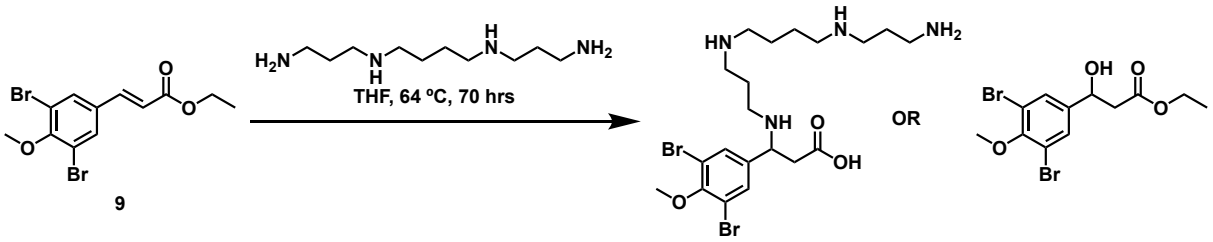
3.5 Exploration of Direct Amidation to form Ianthelliformisamines A and C

We hypothesized that the ianthellilformisamines would be easier to purify if they were formed through a direct amidation because the coupling reagents and intermediates present in the amide coupling reactions would not be needed. Hillgren et al. used an amidation to form the amide in the total synthesis of spermatinamine, a natural product structurally similar to ianthelliformisamine C originally isolated from the marine sponge *Pseudoceratina* sp. (**Scheme 12**).[22]



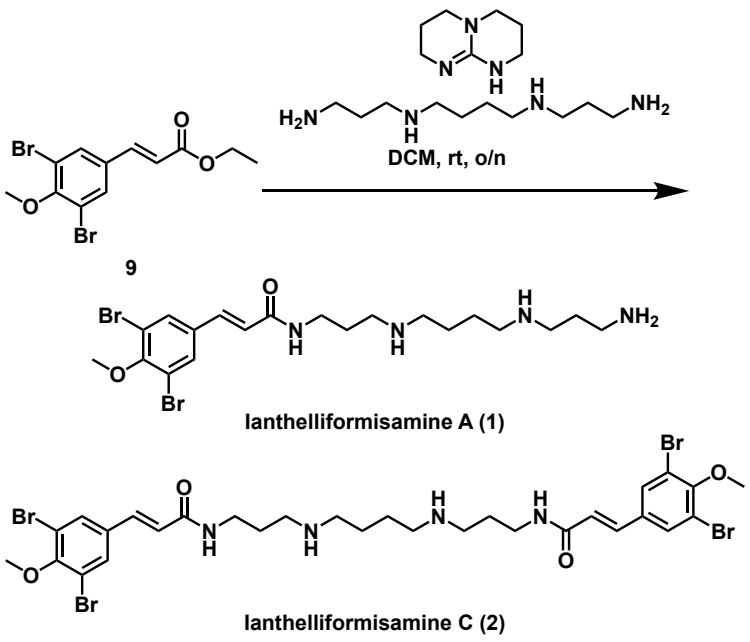
Scheme 12. Transamination used in the synthesis of spermatinamine by Hillgren et al.

Notably, spermatinamine lacks the α-β unsaturated amide present in **1** and **2**, and thus the carboxylic acid precursor is likely a better electrophile capable of reacting with the spermine derivative more efficiently than **10**. This may explain why our amidation reactions were largely unsuccessful for the synthesis of **1** and **2**.



Scheme 13. The attempted amidation of **9** with spermine in THF did not produce **1** or **2** but yielded a Michael addition product.

A series of amidation reactions with different solvent conditions were performed to investigate conditions for the reaction. The ethyl ester **9** and spermine were dissolved in DCM, THF, or dioxane and stirred at reflux for three days (38 ºC, 64 ºC, and 98 ºC, respectively), then crude NMR spectra were obtained for the three reactions. In DCM, the formation of **1** and **2** was not observed. In the dioxane reaction, we observed some degradation to unknown products, but the majority of the starting material remained unreacted. Interestingly, the peaks for the alkene hydrogens were not present in the crude NMR spectrum for the reaction in THF, suggesting the formation of a Michael-addition product with either spermine or water (**Scheme 13**).

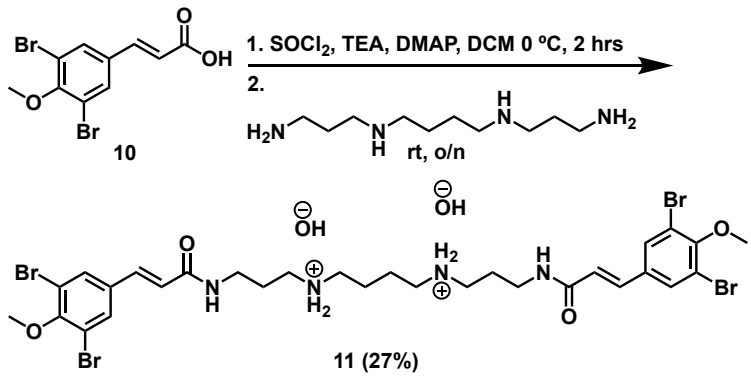


Scheme 14. Amidation between **9** and spermine catalyzed by TBD to produce **1** and **2**.

When performed in methanol, the crude NMR demonstrated the formation of **1** and **2**, however, we were not able to purify **1** and **2** from each other or from the unreacted ethyl ester **(9)** and spermine. When triazabicyclodecene (TBD) was added to the amidation as a catalyst, a small amount of **1** and **2**

was formed, but we were not able to purify it (**Scheme 14**).

3.6 Optimization of Amide Formation Through an Acyl Chloride Intermediate



Scheme 15. Synthesis of protonated ianthelliformisamine C **(11)** through the formation of the acyl chloride derivative of **10.**

Since the amidations were unsuccessful for the synthesis and isolation of **1** and **2**, we began searching for another strategy that would activate the carboxylic acid **10** to become a better electrophile but would have fewer intermediates and byproducts than the amide coupling with traditional peptide coupling reagents. This led us to investigate forming the amide through an acyl chloride, which allowed us to isolate protonated ianthelliformisamine C **(11)** in 27% yield **(Scheme 15)**.

Eq. 10	Eq. spermine	Eq. TEA	Eq. DMAP	Eq. $SOCl_2$	Reaction molarity of 10	% yield 1	% yield 2
1.0	1.7	2.0	0.6	1.9	0.03 mM	-	28%
1.0	0.6	2.0	0.6	2.1	0.03 mM	-	8.0%
1.0	5.1	2.0	0.6	1.9	0.03 mM	-	6.0%
1.0	5.8	2.7	0	1.2	0.1 mM	Both products observed in the crude NMR, but unable to purify	

Table 2. Optimization of the synthesis of ianthelliformisamines A and C **(1** and **2)** through varying the equivalents of **10** and spermine.

Following our successful synthesis of **2**, we varied the equivalents of spermine and **10** in attempts to produce only **1** or **2** and optimize the yield **(Table 2)**. Even when more than five equivalents of spermine were used, we were unable to isolate **1**. We realized its high polarity

caused **1** to stay on the baseline in normal-phase column chromatography, even in 45% MeOH, 50% DCM, and 5% NH_4OH eluent. This led us to investigate other purification methods, such as a series of work-ups and extractions, reverse phase column chromatography, and high-pressure liquid chromatography (HPLC).

3.7 Optimization of Purification Methods for Ianthelliformisamine C

A series of acid-base work-ups and extractions were used to separate the components of the crude mixture following the reaction between the activated acyl chloride derived from **10** and spermine. First, the reaction mixture was quenched with 0.6 M hydrochloric acid to protonate the amines of **1, 2,** and unreacted spermine. Then, the mixture was extracted with ethyl acetate to remove any unreacted **10** and TEA. The aqueous layer containing **1, 2,** and spermine was quickly basified with 1 M sodium hydroxide to prevent hydrolysis. Finally, **1** and **2** were extracted out of the aqueous layer with a mixture of ethyl acetate and methanol. However, **1** and **2** are soluble in water, so it was difficult to completely remove them from the aqueous layer. Furthermore, this procedure cannot separate a mixture of **1** and **2** from each other.

Reverse phase column chromatography with C18 Silica and MeOH and 10% aqueous ammonium hydroxide as the solvents was attempted as a means to purify **1** and **2**, but practical considerations prevented this from being a viable option as the low flow rate and long elution time of various reaction components resulted in the column running for up to eight hours.

When we attempted to purify the reaction through HPLC, only starting material was obtained even though the presence of **2** in the crude material was confirmed through liquid chromatography/mass spectroscopy (LC/MS) and NMR spectroscopy. We hypothesize that the eluent, a mixture of water with 0.1% formic acid and acetonitrilie with 0.1% formic acid, may have caused **2** to undergo an acid-catalyzed hydrolysis to return to the carboxylic acid **10**. We

obtained the same results when attempting HPLC purification of the ianthelliformisamines with a mixture of water with 0.1% ammonium hydroxide and acetonitrile with 0.1% ammonium hydroxide as the eluent, and we propose that either residual acid on the column still caused the hydrolysis to occur, or the sample was too dilute for the UV detector to register the presence of **2**.

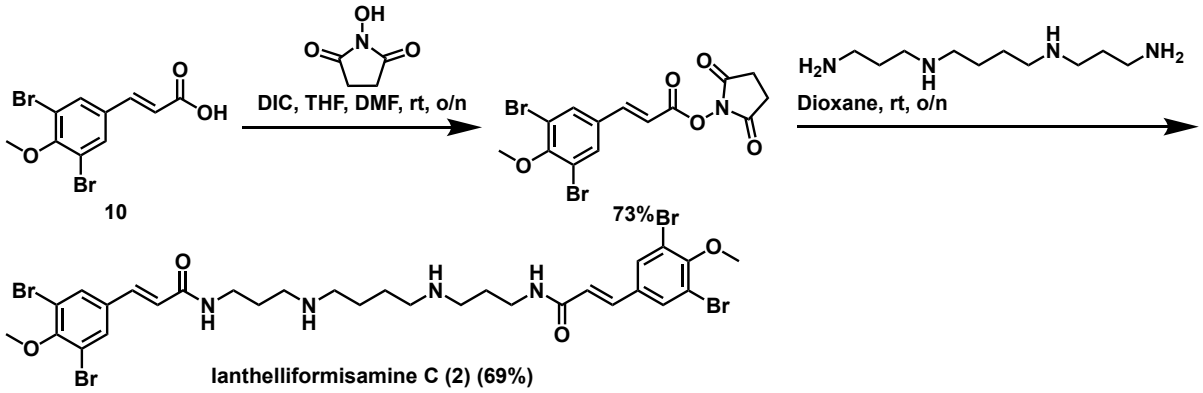
As such, normal phase column chromatography with DCM, MeOH, and ammonium hydroxide remained the leading method to purify **2**. Because it was difficult to isolate **1** from either a peptide coupling or acyl chloride reaction with spermine with any purification method that we tried, we decided to shift our objectives from synthesizing both **1** and **2** to focusing on **2**. Not only is **2** easier to synthesize and purify, but it exhibits broad-spectrum antimicrobial activity and has a significantly higher potency against *P. aeruginosa*, making it a more attractive target for the development of novel antimicrobials.[17]

3.8 DIC Coupling to Synthesize Ianthelliformisamine C in High Yield

Returning to amide coupling, we hypothesized that we could obtain **2** with an easy purification by isolating an activated ester derived from **10** prior to reaction with spermine. Putting this into practice, **2** was synthesized in relatively high yield through the reaction of spermine with the N-hydroxysuccinimide ester derivative of **10 (Scheme 16)**. The ester was isolated through an amide coupling between **10** and n-hydroxysuccinimide (NHS) with diisopropylcarbodiimide (DIC) as the coupling reagent. The isolation of the NHS-ester prior to the reaction with spermine removed unreacted **10** and DIC-coupled activated ester from the purification of **2**. As a result, pure **2** was obtained through basic work-up and extraction without the need for column chromatography or other purification methods.

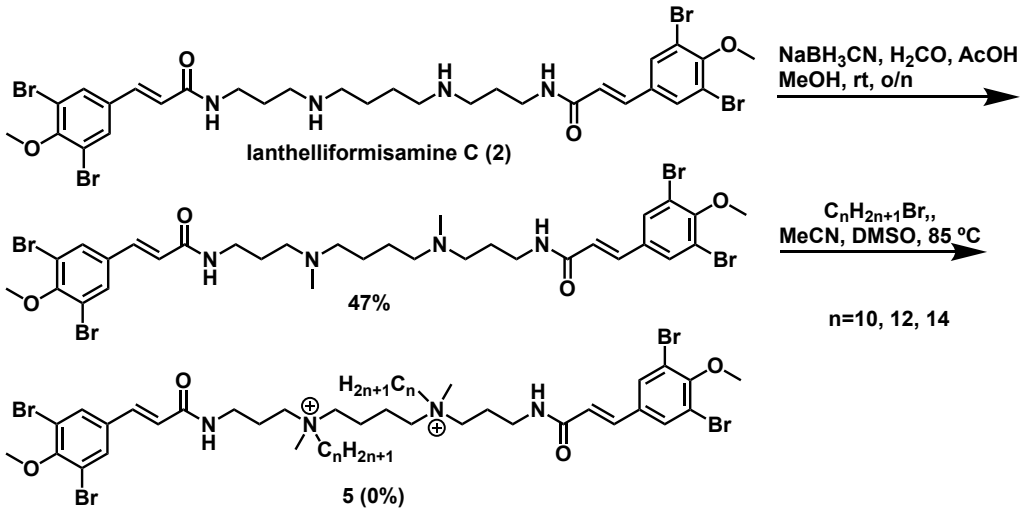
4. Quaternization of Ianthelliformisamine C and Future Work

4.1 Preliminary Results for the Quaternization of Ianthelliformisamines C



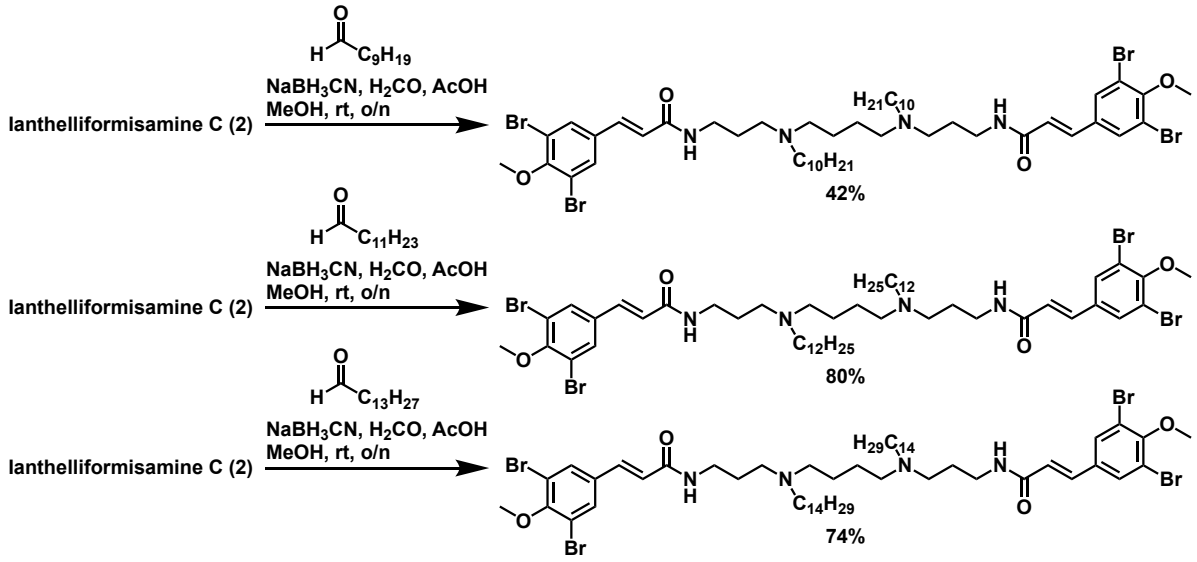
Scheme 16. Formation of **2** through an amide coupling with the NHS-ester of **10**.

2 was methylated through a reductive amination in 47% yield. As mono-methylated products were also observed, we predict that the yield of this reaction may be increased by using a greater number of equivalents of sodium cyanoborohydride, formaldehyde, and acetic acid. Three quaternization reactions were attempted in parallel on a 10 mg scale by stirring **2** with 10-, 12-, and 14-carbon alkyl bromides in dimethylformamide with sodium iodide at 85 ºC for multiple days, but no conversion of the starting material was observed **(Scheme 17)**. We believe the steric



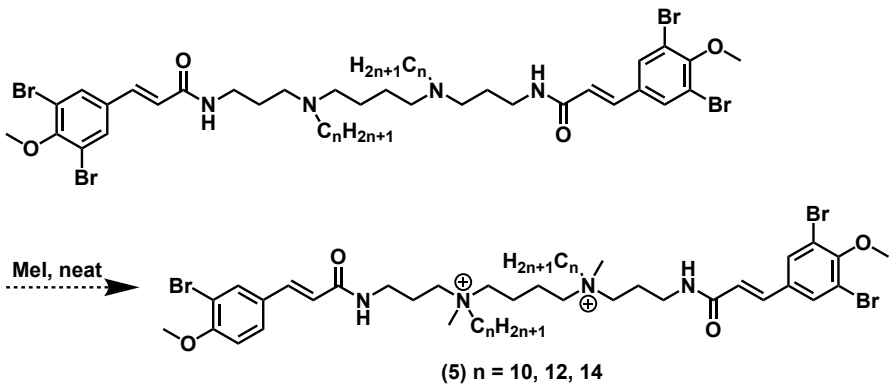
Scheme 17. Methylation and quaternization of the amines in ianthelliformisamine C to generate QACs.

hinderance from the bulky acrylamide groups may be inhibiting the alkyl bromides from reacting with the amines, as internal tertiary amines are typically difficult to quaternize.[23]



Scheme 18. The amines of **2** were be alkylated through a reductive amination with long chain aldehydes in medium-to-high yield.

Going forward, we decided to reverse the order of the quaternization steps, alkylating the secondary amines first when there is less steric hinderance and then methylating to quaternize the amines. The secondary amines were successfully alkylated with alkyl bromides through reductive amination with long-chain alkyl aldehydes **(Scheme 18)**. For the final step of the synthesis, the tertiary amines will be methylated and quaternized with methyl iodide, a much smaller nucleophile than the bromo alkyl chains to produce the QAC products **(Scheme 19)**. The reaction of tertiary amines with neat methyl iodide has been successfully demonstrated with difficult to quaternize compounds.[23]



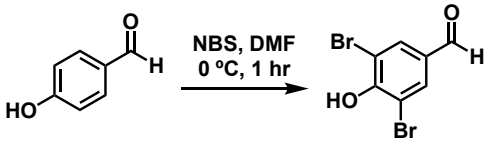
Scheme 19. The alkylated ianthelliformisamine C derivatives will be methylated and quaternized through reaction with methyl iodide.

4.2 Proposed Biological Studies

Minnimum inhibitory concentration assays will be performed on the three QAC analogs and **2** with several clinically-relevant species of bacteria, including *S. aureus* and *P. aeruginosa* to determine the bioactivity and potency of the ianthelliformisamine-derivde QACs. Because QACs act by disrupting cellular membranes and lysing bacterial cells, it is important to ensure they do not easily lyse human cells. As a preliminary test of toxicity, hemolysis assays will be performed on the novel QACs as well.

5. Experimental

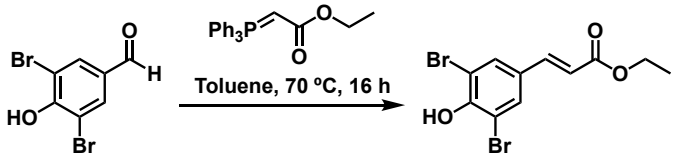
5.1 General Procedure for the Synthesis of Ianthelliformisamine C



3-5-dibromo-4-hydroxybenzaldehyde (7): In a flame dried 25 mL flask under argon was dissolved 4-hydroxybenzaldehyde (1.0013 g, 1.00 eq., 8.1993 mmol) in 3 mL of dimethylformamide. The reaction was cooled to 0 ºC, then recrystallized N-bromosuccinimide (3.0556 g, 2.09 eq., 17.167 mmol) dissolved in 7 mL of dimethylformamide was added dropwise, causeing the reaction to turn from yellow to dark red, then bright orange. The reaction was quenched after one hour with deionized water, and the aqueous layer was extracted with diethyl ether. The organic layer was washed once with deionized water, once with 10% aqueous sodium thiosulfate, and seven times with brine. The organic layers were dried over magnesium sulfate, filtered, and concentrated *in vacuo*, yielding **7** (2.0237g, 88%). Collected spectra matched literature reported spectra.[24]

^{1}H NMR (400 MHz, Acetone) δ 10.31 (s, 1H), 8.54 (s, 2H).

^{13}C NMR (126 MHz, acetone) δ 205.30, 188.46, 133.66, 111.08.

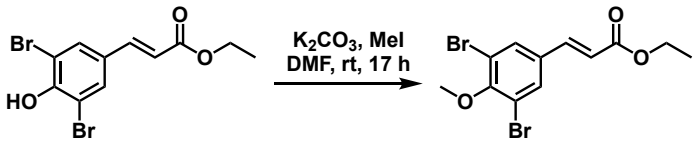


(E)-Ethyl 3-(3,5-dibromo-4-hydroxyphenyl)acrylate (8): In a flame-dried 50 mL flask under argon was dissolved 3-5-dibromo-4-hydroxybenzaldehyde (251.0 mg, 1.00 eq., 0.8967 mmol) in 5 mL of tolune. To this was added carbethoxymethylene triphenylphosphorane (413.6 mg, 1.32 eq., 1.187 mmol). The reaction was stirred at 70 ºC for 16 hours, then evaporated to yield an

orange-brown waxy solid. The crude mixture was purified by column chromatography in 100% diethyl ether, yielding **8** (0.2951 g, 94%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 7.63 (s, 2H), 7.49 (d, 16 Hz, 1H), 6.32 (d, 16 Hz, 1H), 6.07 (s, 1H), 4.26 (q, 7.1 Hz, 2H), 1.33 (t, 7.1 Hz, 3H).

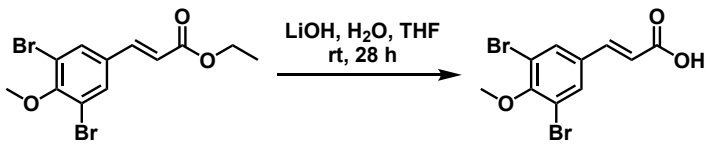
^{13}C NMR (126 MHz, $cdcl_3$) δ 166.88, 154.50, 141.47, 131.56, 125.12, 123.13, 118.19, 110.37, 51.83.



(E)-Methyl 3-(3,5-dibromo-4-methoxyphenyl)acrylate (9): A 100 mL flask charged with potassium carbonate (2.1423 g, 1.12 eq., 15.501 mmol) was flame dried then put under argon. To this was added ethyl-3-(3,5-dibromo-4-hydroxyphenyl)acrylate (4.6626 g, 1.00 eq., 13.878 mmol) dissolved in 25 mL of dimethylformamide then methyl iodide (1.05 mL, 1.22 eq, 16.9 mmol). The reaction was stirred at room temperature for 17 hours then quenched with 30 mL of 1 M sodium hydroxide. The quenched mixture was stirred for an additional 20 minutes, then extracted with ethyl acetate. The organic layer was washed once with 1 M sodium hydroxide, then seven times with brine, dried over magnesium sulfate, filtered, and concentrated *in vacuo* to yield **9** (4.6568 g, 956). All collected spectra matched literature reported spectra.[17]

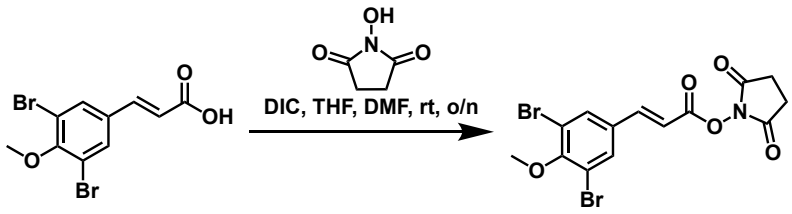
^{1}H NMR (600 MHz, $CDCl_3$) δ 7.66 (s, 2H), 7.50 (d, J=16 Hz, 1H), 6.36 (d, J=15 Hz, 1H), 4.26 (q, J=7.2 Hz, 2H), 3.91 (s, 3H), 1.33 (t, J=7.1 Hz, 3H)

^{13}C NMR (126 MHz, acetone) δ 167.15, 156.31, 141.90, 134.68, 133.37, 121.08, 119.30, 61.25, 52.06.



(E)-3-(3,5-dibromo-4-methoxyphenyl)acrylic acid (10): In a 25 mL flask was dissolved methyl (E)-3-(3,5-dibromo-4-methoxyphenyl)acrylate (1.2208 g, 1.00 eq., 3.4879 mmol) and lithium hydroxide (0.3343 g, 4.00 eq., 13.96 mmol) in 7 mL of tetrahydrofuran and 2 mL of deionized water. The reaction was stirred at room temperature open to air for 28 hours, then quenched with 5 mL of 1 M hydrochloric acid and extracted with ethyl acetate. The organic layer was washed with brine, dried over magnesium sulfate, filtered, and concentrated *in vacuo*. The crude mixture was purified with normal phase chromatography, using a gradient of ethyl acetate with 0.1% acetic acid in hexanes. A fraction containing **10** (0.9724 g, 83%) was eluted at 10% ethyl acetate with 0.1% acetic acid. All collected spectra matched literature reported spectra.[17]

^{1}H NMR (500 MHz, acetone) δ 7.99 (s, 2H), 7.59 (d, J=16 Hz, 1H), 6.62 (d, J=16 Hz, 1H), 3.90 (s, 3H).



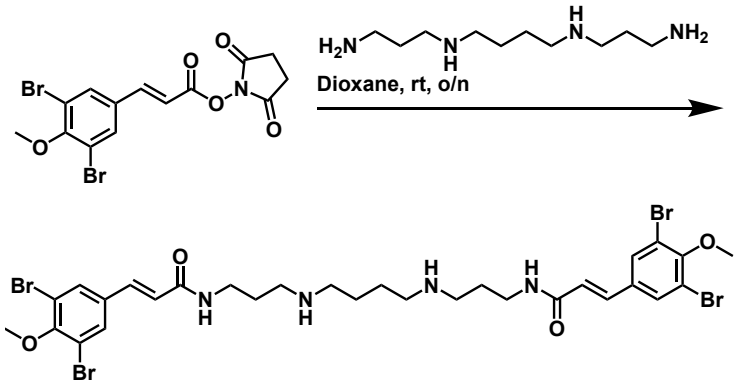
2,5-dioxopyrrolidin-1-yl (E)-3-(3,5-dibromo-4-methoxyphenyl)acrylate: In a flame-dried flask under argon was dissolved (E)-3-(3,5-dibromo-4-methoxyphenyl)acrylic acid (2.5080 g, 1.00 eq., 7.4647 mmol) in 30 mL of tetrahydrofuran and 3.0 mL of dimethylformamide. N-N'-diisopropylcarbodiimide (1.1305 g, 1.5 eq., 8.9577 mmol) and N-hydroxysuccinimide (1.1007 g, 1.28 eq., 9.5638 mmol) were added, and the reaction was stirred at room temperature overnight. The crude products were filtered, concentrated *in vacuo,* and purified with normal phase

chromatography using a gradient of 0% to 10% ethyl acetate in hexanes to yield 2,5-dioxopyrrolidin-1-yl (E)-3-(3,5-dibromo-4-methoxyphenyl)acrylate (2.3580 g, 73%).

^{1}H NMR (600 MHz, $CDCl_3$) δ 7.72 (m, 3H), 6.52 (d, J=16 Hz, 1H), 3.93 (s, 3H), 2.89 (s, 4H).

^{13}C NMR (101 MHz, $CDCl_3$) δ 169.27, 161.55, 156.64, 146.28, 132.69, 132.10, 119.11, 113.58, 60.97, 25.74.

MS: Accurate Mass (ESI-) found: 465.86981, $C_{14}H_{11}O_5N^{79}Br_2{}^{35}Cl$ ($M+Cl^-$) requires 465.8692.

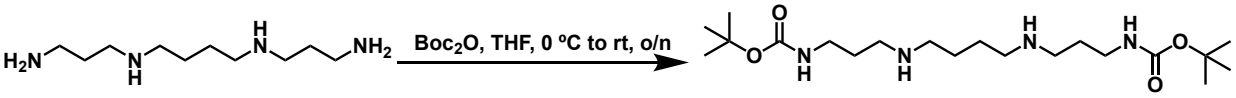


Ianthelliformisamine C (2): In a flame-dried flask under argon was dissolved 2,5-dioxopyrrolidin-1-yl (E)-3-(3,5-dibromo-4-methoxyphenyl)acrylate (1.9978 g, 2.31 eq., 4.6133 mmol). Spermine (403.4 mg, 1.00 eq., 1.994 mmol) was added, and the reaction was stirred at room temperature overnight. The reaction mixture was quenched with saturated aqueous sodium bicarbonate. The organic layer was diluted with dichloromethane and extracted three times with saturated aqueous sodium bicarbonate. The combined aqueous layers were basified to a pH 14 using 1 M sodium hydride, then extracted five times with 10% methanol in dichloromethane. The combined organic layers were dried over sodium sulfate, filtered, and concentrated *in vacuo* to obtain pure **2** (1.1570 g, 69%), a pale yellow solid. All collected spectra matched literature reported spectra.[17]

^{1}H NMR (500 MHz, CD_3OD) δ 7.77 (s, 4H), 7.38 (d, J=16 Hz, 2H), 6.55 (d, J=16 Hz, 2H), 3.87 (s, 6H), 3.38 (t, 6.9 Hz, 4H), 2.72 (m, 8H), 1.82 (m, 4H), 1.66 (m, 4H).

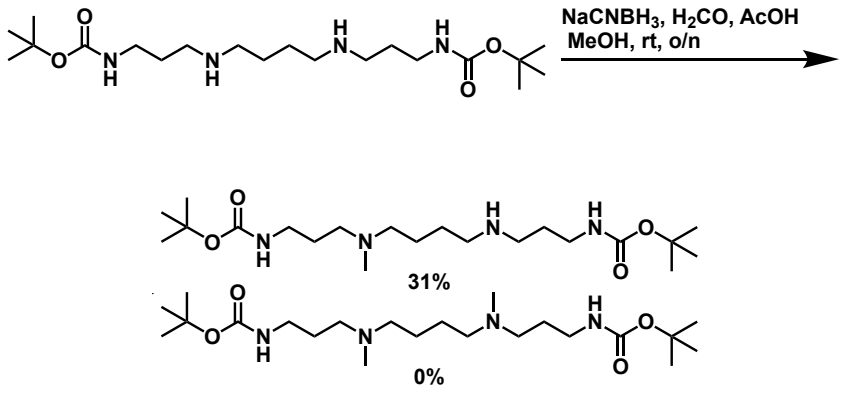
^{13}C NMR (101 MHz, DMSO) δ 164.33, 153.75, 134.83, 134.50, 131.47, 124.71, 117.96, 60.50, 49.42, 46.96, 37.10, 29.46, 27.57.

5.2 General Procedures for the Methylation and Alkylation of Spermine and Ianthelliformisamine C



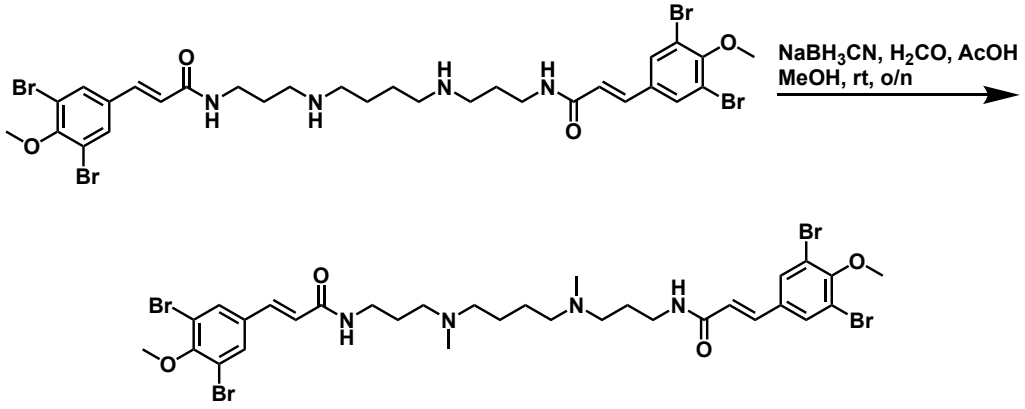
Di-tert-butyl ((butane-1,4-diylbis(azanediyl))bis(propane-3,-diyl))dicarbamate: In a flame-dried flask under argon was dissolved spermine (498.6 mg, 1.00 eq., 2.464 mmol) in 7 mL tetrahydrofuran. The flask was cooled to 0 ºC and Boc anhydride (543.4 mg, 1.01 eq., 2.490 mmol) dissolved in 5 mL tetrahydrofuran was added dropwise. The reaction was stirred for 18 hours starting at 0 ºC and warming to room temperature, then concentrated *in vacuo* to afford a colorless oil. Normal phase column chromatography with a gradient of methanol with 10% ammonium hydroxide and DCM was performed to obtain pure di-tert-butyl ((butane-1,4-diylbis(azanediyl))bis(propane-3,-diyl))dicarbamate (171.1 mg, 17%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 3.19 (m, 4H), 2.64 (m, 8H), 2.08 (m, 7H), 1.66 (m, 4H), 1.44 (s, 2H), 1.43 (s, 17H).



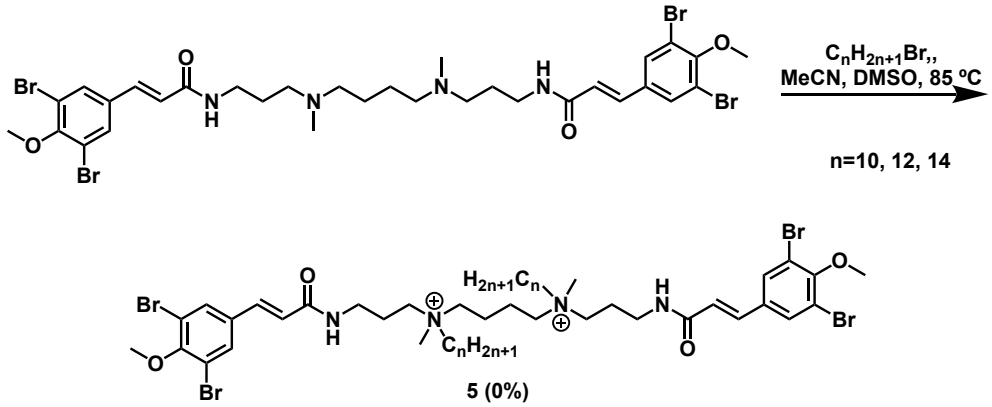
Tert-butyl (2,2,14-trimethyl-4-oxo-3-oxa-5,9,14-triazaheptadecan-17-yl) carbamate: In a vial under argon was dissolved di-tert-butyl ((butane-1,4-diylbis(azanediyl))bis(propane-3,-diyl))dicarbamate (53.9 mg, 1.00 eq., 0.134 mmol) in 2 mL of methanol. To this was added acetic acid (0.25 mL, 33 eq., 4.4 mmol), formaldehyde (37% aqueous) (30 µL, 3.0 eq., 0.4 mmol), and sodium cyanoborohydride (28.3 mg, 3.36 eq., 0.45 mmol), and the reaction was stirred at room temperature for 18 hours. The reaction was quenched with 3 mL of 2 M NaOH, extracted thrice with ethyl acetate, washed with brine, dried with sodium sulfate, filtered, and concentrated *in vacuo*. Normal phase column chromatography was performed with 1% ammonium hydroxide, 9% methanol, and 90% dichloromethane to obtain tert-butyl (2,2,14-trimethyl-4-oxo-3-oxa-5,9,14-triazaheptadecan-17-yl) carbamate (17.3 mg, 31%).

^{1}H NMR (500 MHz, $CDCl_3$) δ 5.29 (s, 1H), 4.82 (s, 2H), 3.58 (s, 3H), 3.17 (m, 6H), 2.54 (m, 6H), 2.38 (m, 3H), 2.21 (s, 2H), 1.64 (m, 5H), 1.43 (s, 20H), 1.25 (m, 2H).

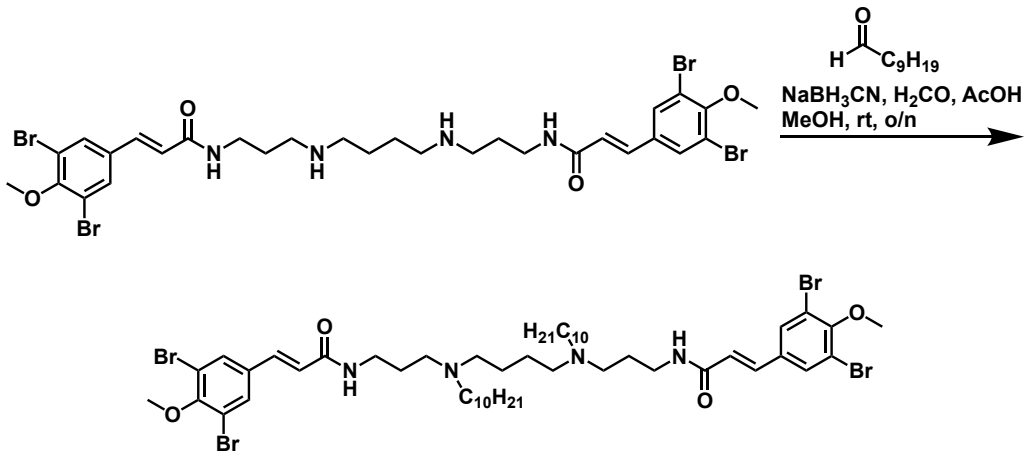


(2E,2'E)-N,N'-((butane-1,4-diylbis(azanediyl))bis(propane-3,1-diyl))bis(3-(3,5-dibromo-4-methoxyphenyl)acrylamide): Ianthelliformisamine C (332.3 mg, 1.000 eq., 396.4 mmol) was dissolved in 4.0 mL of methanol. To this was added formaldehyde (37% aqueous) (73.79 µL, 2.5 eq., 0.9910 mmol), acetic acid (113.5 µL, 5.00 eq., 1.982 mmol), and sodium cyanoborohydride (52.4 mg, 2.10 eq., 0.834 mmol). The reaction was stirred at room temperature overnight then extracted with ethyl acetate. Normal phase column chromatography with a gradient of methanol,

dichloromethane, and ammonium hydroxide was performed to obtain pure 2E,2'E)-N,N'-((butane-1,4-diylbis(azanediyl))bis(propane-3,1-diyl))bis(3-(3,5-dibromo-4-methoxyphenyl)acrylamide) (161.2 mg, 47%).

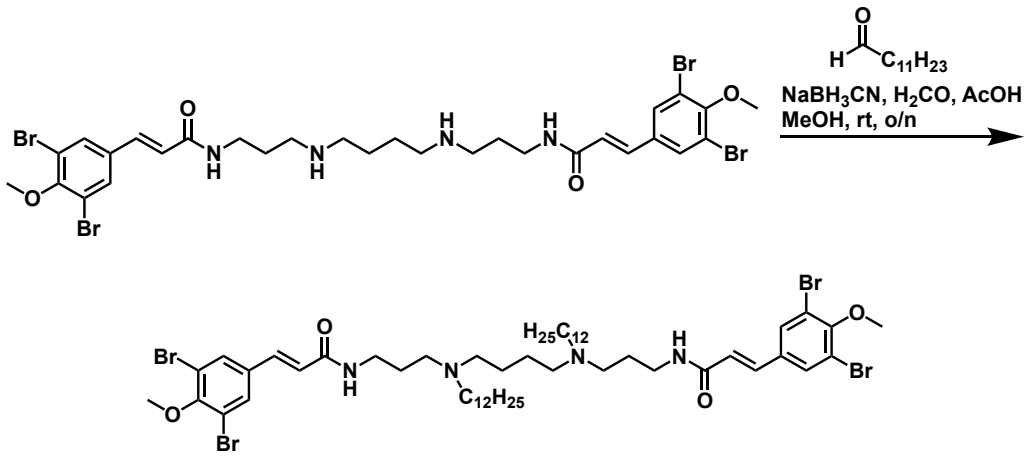


General procedure for the alkylation of (2E,2'E)-N,N'-((butane-1,4-diylbis(azanediyl))bis(propane-3,1-diyl))bis(3-(3,5-dibromo-4-methoxyphenyl)acrylamide): (2E,2'E)-N,N'-butane-1,4-diylbis(azanediyl))bis(propane-3,1-diyl))bis(3-(3,5-dibromo-4-methoxyphenyl)acrylamide) (9.8188 mg, 1.00 eq., 0.01133 mmol) was dissolved in 1 mL of acetonitrile and 0.125 mL of dimethylsulfoxide. Long chain alkyl bromides (8.40 eq., 0.095203 mmol, 1-bromodecane: 19.75 µL; 1-bromododecane: 22.86 µL; 1-bromotetradecane: 28.3 µL) was added, and the reaction was stirred at 85 ºC for 2 days. No conversion of the starting material was observed. An additional 2.0 equivalents (0.0227 mmol) of the long chain alkyl bromides (1-bromodecane: 4.70 µL; 1-bromododecane: 5.44 µL; 1-bromotetradecane: 6.74 µL) was added, and no conversion of the starting material was observed.



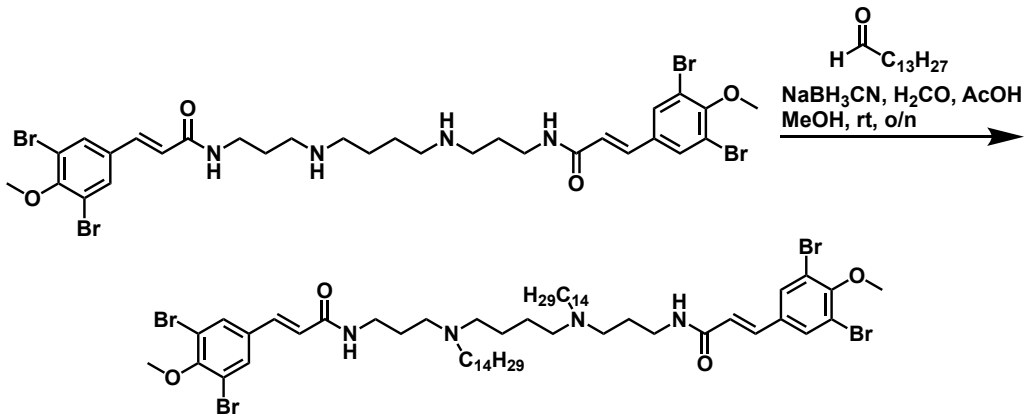
(2E,2'E)-N,N'-((butane-1,4-diylbis(decylazanediyl))bis(propane-3,1-diyl))bis(3-(3,5-dibromo-4-methoxyphenyl)acrylamide): In a flame-dried flask under argon was dissolved ianthelliformisamine C (50.0 mg, 1.00 eq., 0.0596 mmol) in 0.6 mL of methanol and decanal (50.0 µL, 4.45 eq., 0.266 mmol). To this was added acetic acid (18.0 µL, 5.27 eq., 0.314 mmol), and the reaction was stirred at room temperature for 30 minutes. Sodium cyanoborohydride (15.0 mg, 4.00 eq., 0.239 mmol) was added in two portions, and the reaction was stirred at room temperature for 24 hours, then quenched with 1 M sodium hydroxide. The aqueous layer was extracted thrice with dichloromethane and once with ether, then the combined organic layers were dried over sodium sulfate, filtered, and concentrated *in vacuo*. The crude products were purified by column chromatography with 0.5% ammonium hydroxide, 4.5% methanol, and 95% dichloromethane to obtain (2E,2'E)-N,N'-((butane-1,4-diylbis(decylazanediyl))bis(propane-3,1-diyl))bis(3-(3,5-dibromo-4-methoxyphenyl)acrylamide) (28.2 mg, 42%)

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.70 (t, J=5 Hz, 2H), 7.58 (s, 4H), 7.40 (d, J=16 Hz, 2H), 6.29 (d, J=15 Hz, 2H), 3.88 (s, 6H), 3.44 (m, 4H), 2.51 (t, J=6 Hz, 4H), 2.39 (m, 8H), 1.66 (m, 4H), 1.44 (m, 8H), 1.23 (m, 28H), 0.85 (t, J=7 Hz, 6H).



(2E,2'E)-N,N'-((butane-1,4-diylbis(dodecylazanediyl))bis(propane-3,1-diyl))bis(3-(3,5-dibromo-4-methoxyphenyl)acrylamide): In a flame-dried flask under argon was dissolved ianthelliformisamine C (48.6 mg, 1.00 eq., 0.0580 mmol) was dissolved in 0.6 mL of methanol and dodecanal (60 µL, 4.7 eq., 0.27 mmol). To this was added acetic acid (18.0 µL, 5.42 eq., 0.314 mmol), and the reaction was stirred at room temperature for 30 minutes. Sodium cyanoborohydride (14.6 mg, 4.00 eq., 0.232 mmol) was added in two portions, and the reaction was stirred at room temperature overnight, then quenched with 1 M sodium hydroxide. The aqueous layer was extracted thrice with dichloromethane and once with ether, then the combined organic layers were dried over sodium sulfate, filtered, and concentrated *in vacuo*. The crude products were purified by column chromatography with 0.5% ammonium hydroxide, 4.5% methanol, and 95% dichloromethane. The purified fraction containing the alkylated product was redissolved with ethyl acetate and washed with 1 M sodium hydroxide. The organic layer was concentrated *in vacuo* to obtain (2E,2'E)-N,N'-((butane-1,4-diylbis(dodecylazanediyl))bis(propane-3,1-diyl))bis(3-(3,5-dibromo-4-methoxyphenyl)acrylamide) (54.3 mg, 80%).

^{1}H NMR (600 MHz, $CDCl_3$) δ 7.76 (t, J=5 Hz, 2H), 7.57 (s, 4H), 7.40 (d, J=17 Hz, 2H), 6.33 (d, J=16 Hz, 2H), 3.87 (s, 6H), 3.43 (q, J=6 Hz, 4H), 2.54 (m, 4H), 2.43 (m, 8H), 1.69 (m, 4H), 1.46 (m, 8H), 1.21 (m, 34H), 0.85 (t, J=8 Hz, 6H).

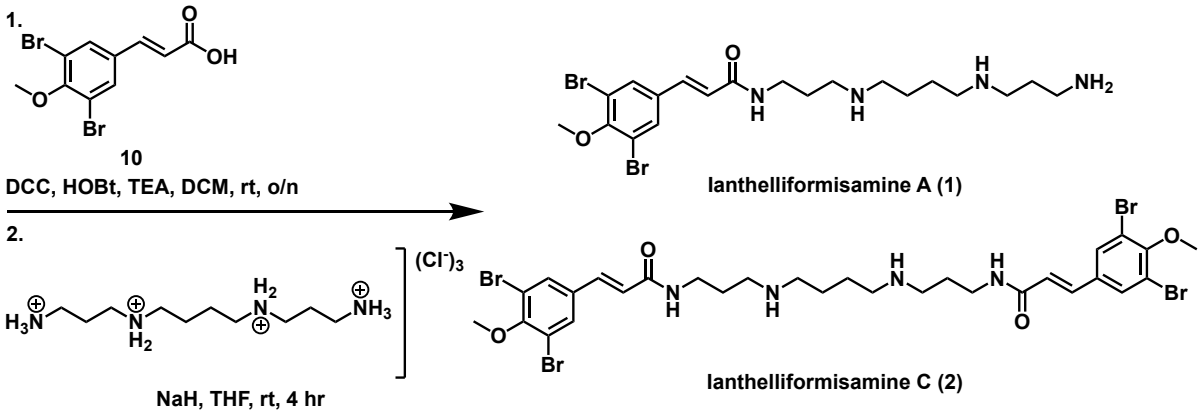


(2E,2'E)-N,N'-((butane-1,4-diylbis(tetradecylazanediyl))bis(propane-3,1-diyl))bis(3-(3,5-dibromo-4-methoxyphenyl)acrylamide): In a flame-dried flask under argon, ianthelliformisamine C (50.6 mg, 1.00 eq., 0.0604 mmol) and dodecanal (55.5 mg, 4.33 eq., 0.261 mmol) were dissolved in 0.6 mL of methanol. To this was added acetic acid (18.0 µL, 5.21 eq., 0.314 mmol), and the flask was lightly heated with a heat gun to dissolve the dodecanal. The reaction was stirred at room temperature for 30 minutes, then sodium cyanoborohydride (14.6 mg, 4.00 eq., 0.232 mmol) was added in two. The reaction was stirred at room temperature overnight, then quenched with 1 M sodium hydroxide. The aqueous layer was extracted thrice with dichloromethane and once with ether, then the combined organic layers were dried over sodium sulfate, filtered, and concentrated *in vacuo*. The crude products were purified by column chromatography with 0.5% ammonium hydroxide, 4.5% methanol, and 95% dichloromethane. The purified fraction containing the alkylated product was redissolved with ethyl acetate and washed with 1 M sodium hydroxide. The organic layer was concentrated *in vacuo* to obtain (2E,2'E)-N,N'-((butane-1,4-diylbis(tetradecylazanediyl))bis(propane-3,1-diyl))bis(3-(3,5-dibromo-4-methoxyphenyl)acrylamide) (74.3 mg, 74%).

^{1}H NMR (400 MHz, $CDCl_3$) δ 7.71 (t, J=5 Hz, 2H), 7.57 (s, 4H), 7.40 (d, J=15 Hz, 2H), 6.29 (d, J=15 Hz, 2H), 3.87 (s, 6H), 3.43 (q, J=6 Hz, 4H), 2.50 (t, J=6 Hz, 4H), 2.38 (m, 8H), 1.67 (m, 4H), 1.44 (m, 8H), 1.23 (m, 44H), 0.86 (t, J=7 Hz, 6H).

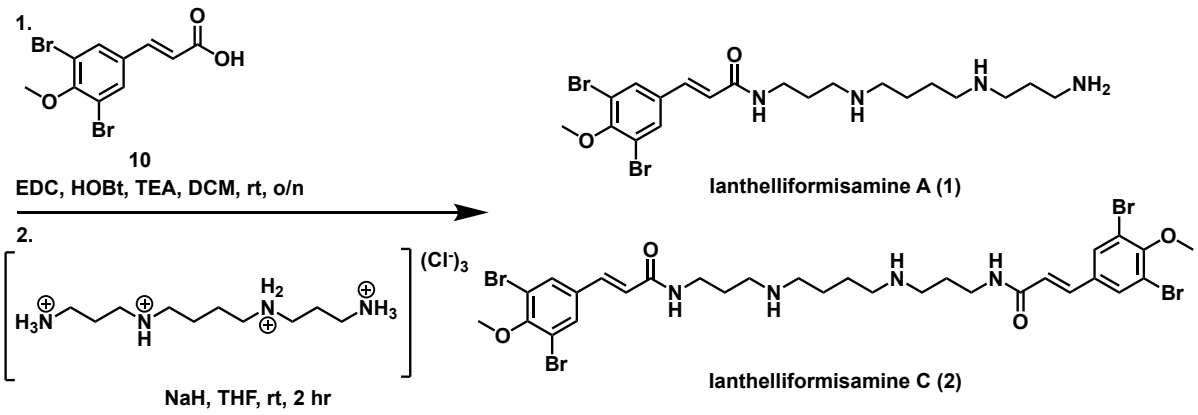
5.3 General Procedures for the Optimization of the Synthesis of Ianthelliformisamine C

DCC Coupling with Spermine Hydrochloride Salt:

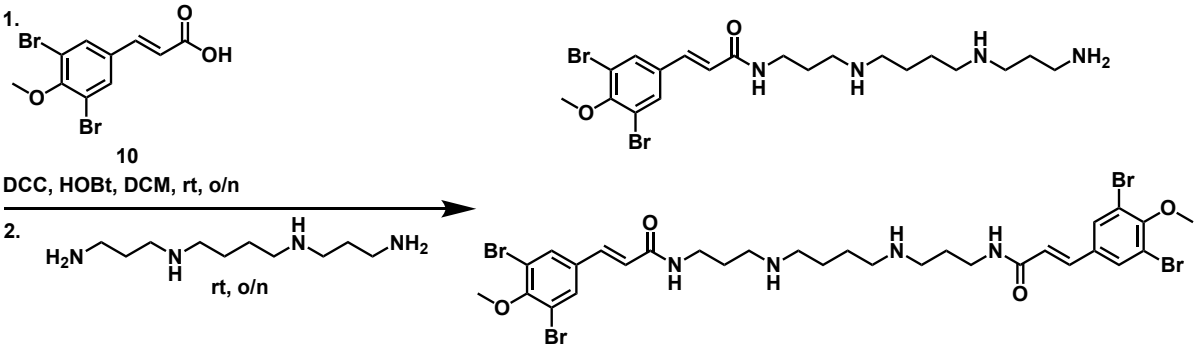


In a flame-dried 10 mL flask under argon was suspended spermine hydrochloride (103.9 mg, 3.96 eq., 298.4 mmol). To this was added 60% sodium hydride emulsion in mineral oil (11.9 mg, 3.95 eq., 0.298 mmol). The reaction was stirred at room temperature for four hours, then filtered and washed with methanol, and the filtrate was concentrated *in vacuo*. In a flame-dried 10 mL flask under argon was suspended 3-(3,5-dibromo-4-methoxyphenyl)acrylic acid (25.3 mg, 1.00 eq., 0.0753 mmol). To this was added N,N-dicyclohexylcarbodiimide (62.6 mg, 4.03 eq, 0.303 mmol), hydroxybenzotriazole (40.0 mg, 3.93 eq., 0.296 mmol), and triethylamine (42 µL, 4.00 eq., 0.301 mmol), and the reaction was stirred at room temperature for five hours. The deprotonated spermine was dissolved in 25 mL dimethylformamide and added to the coupling mixture. The reaction was stirred at room temperature for nineteen hours, then concentrated *in vacuo*. A reverse-phase column with C18 silica was run with a gradient of water and acetonitrile and constant 10% ammonium hydroxide, but the products were not obtained.

EDC coupling with Spermine Hydrochloride Salt



In a flame-dried 10 mL flask under argon was suspended spermine hydrochloride (103.7 mg, 4.07 eq., 297.8 mmol) and 60% sodium hydride emulsion in mineral oil (11.8 mg, 4.0 eq., 0.30 mmol) in 2 mL. The reaction was stirred at room temperature for one hour. In a flame-dried 10 mL flask under argon was suspended 3-(3,5-dibromo-4-methoxyphenyl)acrylic acid (24.6 mg, 1.00 eq., 0.0732 mmol) in 2 mL dichloromethane. To this was added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (46.0 mg, 4.05 eq, 0.296 mmol), hydroxybenzotriazole (39.6 mg, 4.00 eq., 0.293 mmol), and triethylamine (40. µL, 3.9 eq., 0.29 mmol), and the reaction was stirred at room temperature for two hours. The spermine solution was transferred into the coupling reaction, and this was stirred at room temperature for 23 hours, then evaporated to dryness. Normal phase column chromatography was performed on the crude mixture with dichloromethane, isopropanol, and 10% ammonium hydroxide. Only starting materials were collected.

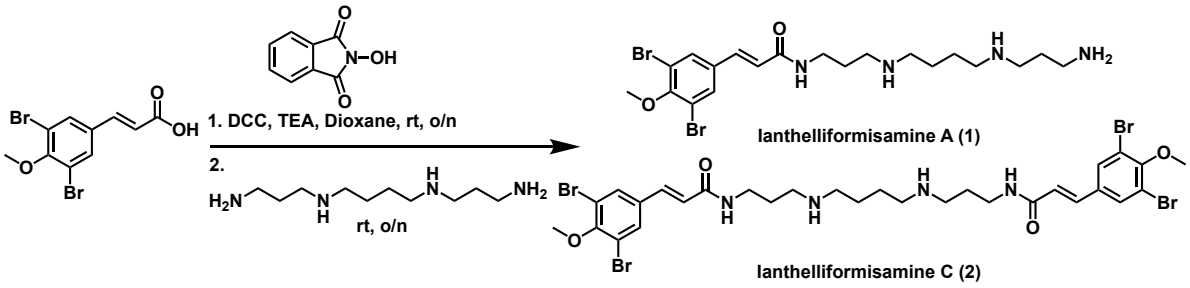


DCC Coupling (Trial 1): In a flame-dried 250 mL flask under argon was dissolved 3-(3,5-dibromo-4-methoxyphenyl)acrylic acid **(10)** (369.9 mg, 1.00 eq., 1.101 mmol) in 30 mL of dichloromethane. To this was added N,N-dicyclohexylcarbodiimide (249.4 mg, 1.10 eq, 1.209 mmol) and hydroxybenzotriazole (184.1 mg, 1.24 eq., 1.362 mmol), and the reaction was stirred at room temperature for 15 hours. The reaction mixture was filtered into a flame-dried 250 mL flask and washed with 15 mL of DCM. Spermine (248 mg, 1.11 eq., 1.23 mmol) was added to the filtrate. The reaction was stirred at room temperature for 18 hours, then concentrated *in vacuo*. Normal phase column chromatography was performed with a gradient of methanol, dichloromethane, and a constant 10% ammonium hydroxide, and an impure fraction containing ianthelliformisamine C **(2)** was obtained. Two more successive normal phase columns were run on the mixed fraction to isolate **2** (134.9 mg, 29%).

DCC Coupling-Trial 2: In a flame-dried flask under argon was dissolved 3-(3,5-dibromo-4-methoxyphenyl)acrylic acid **(10)** (924.0 mg, 1.00 eq., 2.750 mmol) in 54 mL dichloromethane. To this was added N,N-dicyclohexylcarbodiimide (617.2 mg, 1.01 eq, 2.991 mmol) and hydroxybenzotriazole (502.7 mg, 1.19 eq., 3.283 mmol), and the reaction was stirred at room temperature for 17 hours. More N,N-dicyclohexylcarbodiimide (287.5 mg, 0.47 eq, 1.39 mmol) was added, and the reaction was stirred for another five hours. The coupling reaction mixture was filtered and washed with 10 mL of dichloromethane, and spermine (667.8 mg, 1.2 eq., 3.300 mmol) was added to the filtrate. The reaction was stirred at room temperature overnight, then concentrated *in vacuo*. Normal phase column chromatography was run on the crude material with a gradient of methanol, dichloromethane, and ammonium hydroxide. A fraction containing Ianthelliformisamine C **(2)** and hydroxybenzotriazole was obtained. This was dissolved in ethyl acetate and acetone and extracted three times with 1 M sodium hydroxide. The combined organic

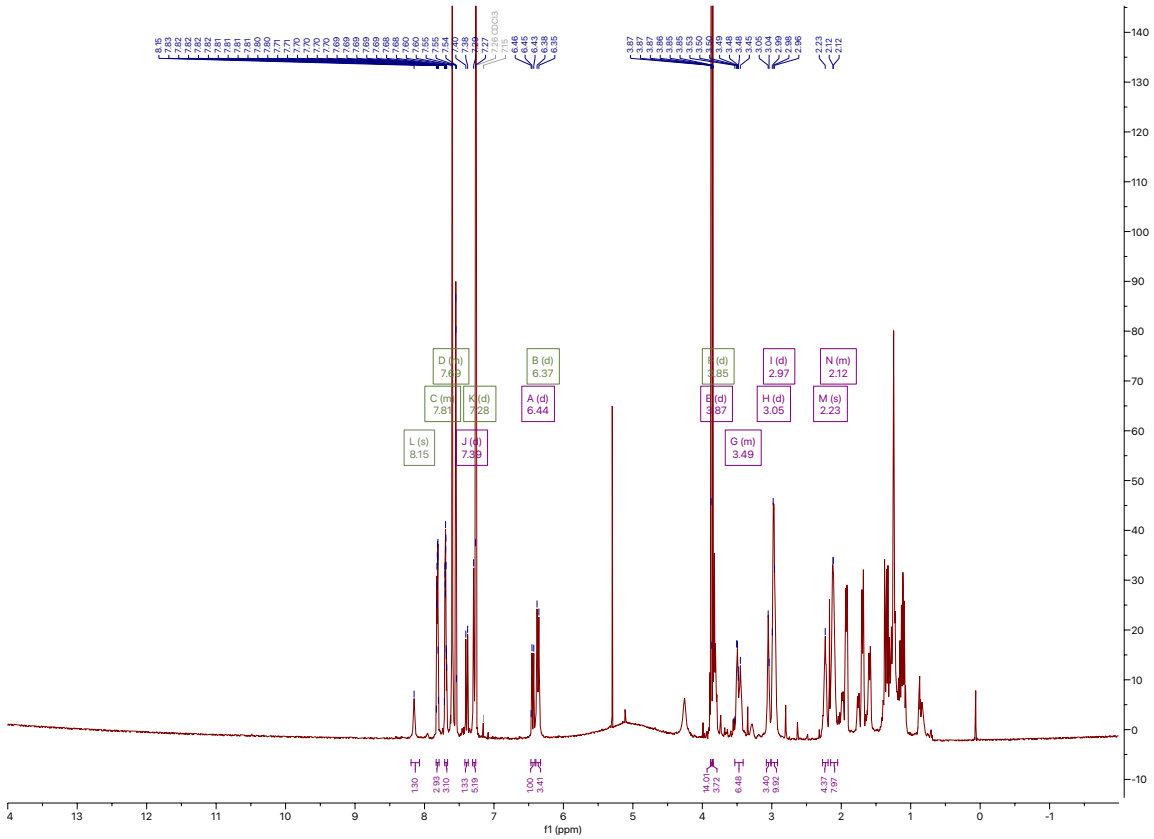
layers were dried over magnesium sulfate, filtered through celite, and concentrated *in vacuo* to yield **2** (78.5 mg, 3%). Another fraction from the column contained a mixture of Ianthelliformisamine A **(1)**, **2** and spermine, and another column was performed to isolate **1** (202.9 mg, 14%). All spectra match literature reported data.[17]

Ianthelliformisamine A: ^{1}H NMR (500 MHz, CD_3OD) δ 7.80 (s, 1H), 7.39 (d, J=16 Hz, 1H), 6.56 (d, J=16 Hz, 1H), 3.88 (s, 3H), 3.36 (t, 2H), 2.64 (m, 12H), 1.78 (m, 4H), 1.55 (m, 4H).

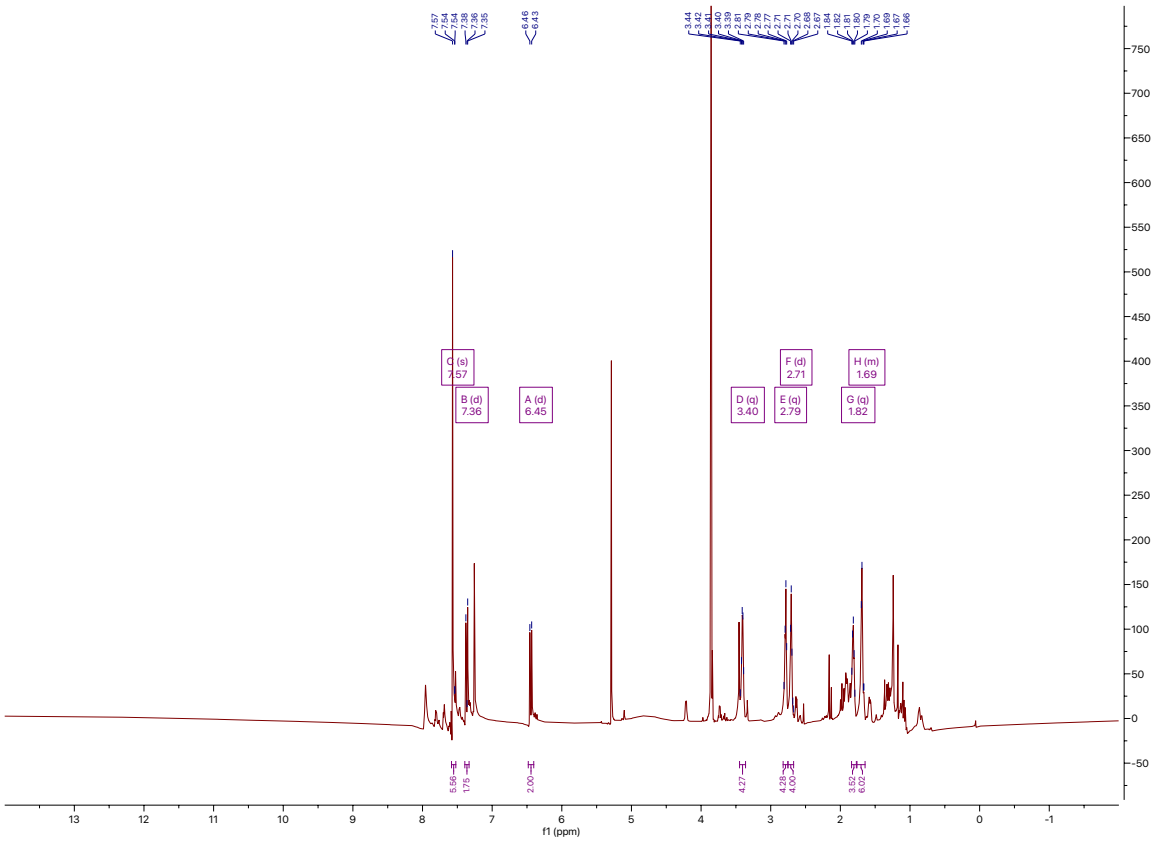


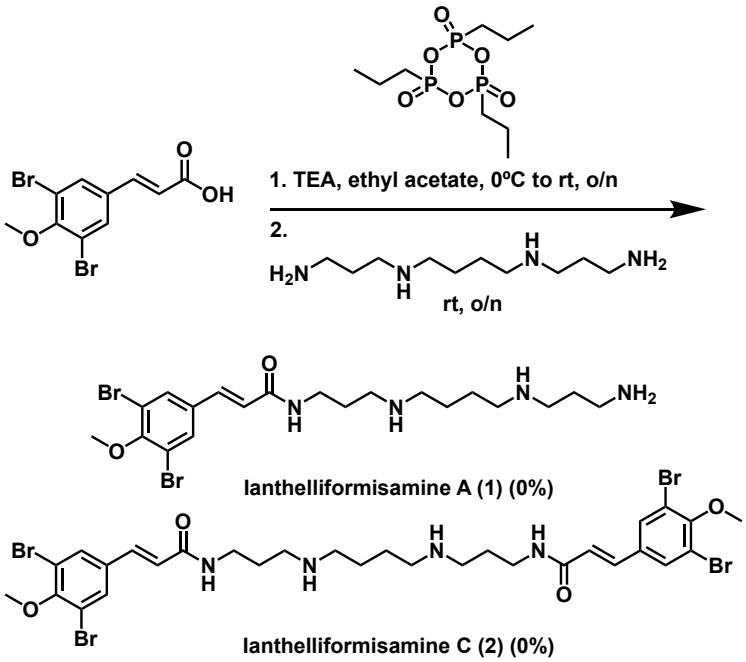
DCC Coupling with Hydroxyphthalimide: In a flame-dried 25 mL flask under agon was dissolved (E)-3-(3,5-dibromo-4-methoxyphenyl)acrylic acid **(10)** (49.9 mg, 1.00 eq., 0.149 mmol) in 7 mL. To this was added N,N-dicyclohexylcarbodiimide (36.7 mg, 1.20 eq, 0.178 mmol) and hydroxyphthalimide (37.9 mg, 1.56 eq., 0.232 mmol), the reaction was stirred at room temperature for five hours. Spermine (33.3 mg, 1.11 eq., 0.165 mmol) and triethylamine (50 µL, 2.4 eq., 0.36 mmol) were added to the flask, and the reaction was stirred at room temperature for 17 hours. At this point, thin-layer chromatography showed remaining N,N-dicyclohexylcarbodiimide- and hydroxyphthalimide-coupled activated ester intermediates. More spermine (26.7 mg, 0.89 eq., 0.132 mmol) was added, and the reaction was stirred at room temperature for another two hours, then concentrated *in vacuo*. Normal phase column chromatography was performed with a gradient of dichloromethane and 10% ammonium hydroxide in methanol.

A fraction was collected containing a mixture of Ianthelliformisamine A (annotated in purple) and hydroxyphthalimide (annotated in green) (11.6 mg):

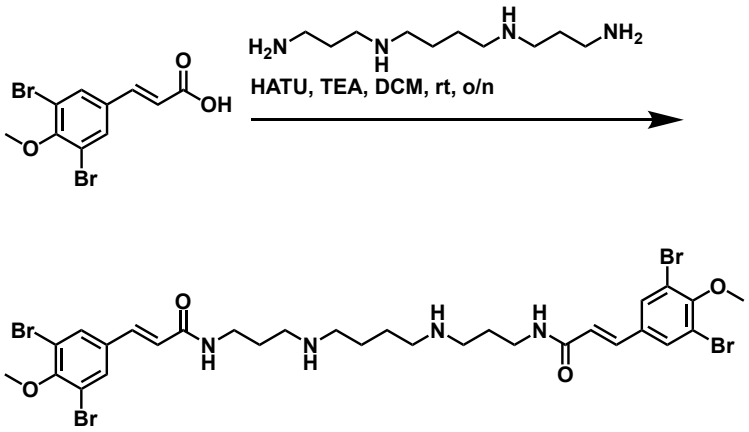


Another fraction was collected containing Ianthelliformisamine C (annotated in purple) and other impurities (34.7 mg):



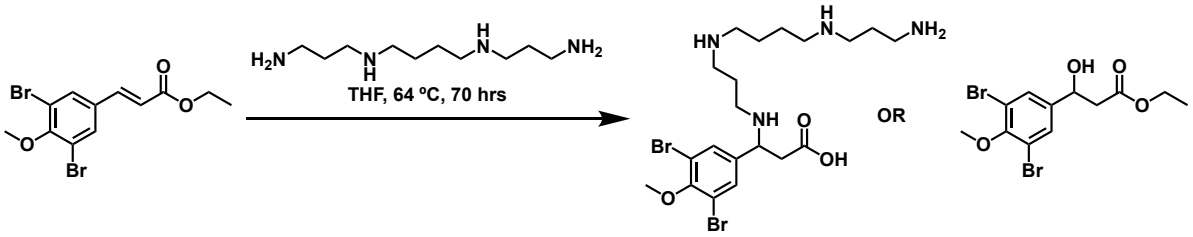


T3P Amide Coupling: In a flame-dried flask under argon was dissolved 3-(3,5-dibromo-4-methoxyphenyl)acrylic acid **(10)** (53.1 mg, 3.20 eq., 0.158 mmol) and triethylamine (30.0 µL, 4.36 eq., 0.215 mmol) in 0.5 mL ethyl. The reaction was cooled to 0 ºC, propanephosphonic acid anhydride (50% in ethyl acetate, 0.12 mL, 4.12 eq., 0.204 mmol) was added, then the ice bath was removed and the reaction was stirred at room temperature for 15 minutes. To this was added Spermine (10.0 mg, 1.00 eq., 0.0494 mmol), and the reaction was stirred at room temperature overnight. No product formation was observed. The reaction was stirred at 45 ºC for another 8 hours, then at 65 ºC for another two days, yielding no conversion of the starting materials.

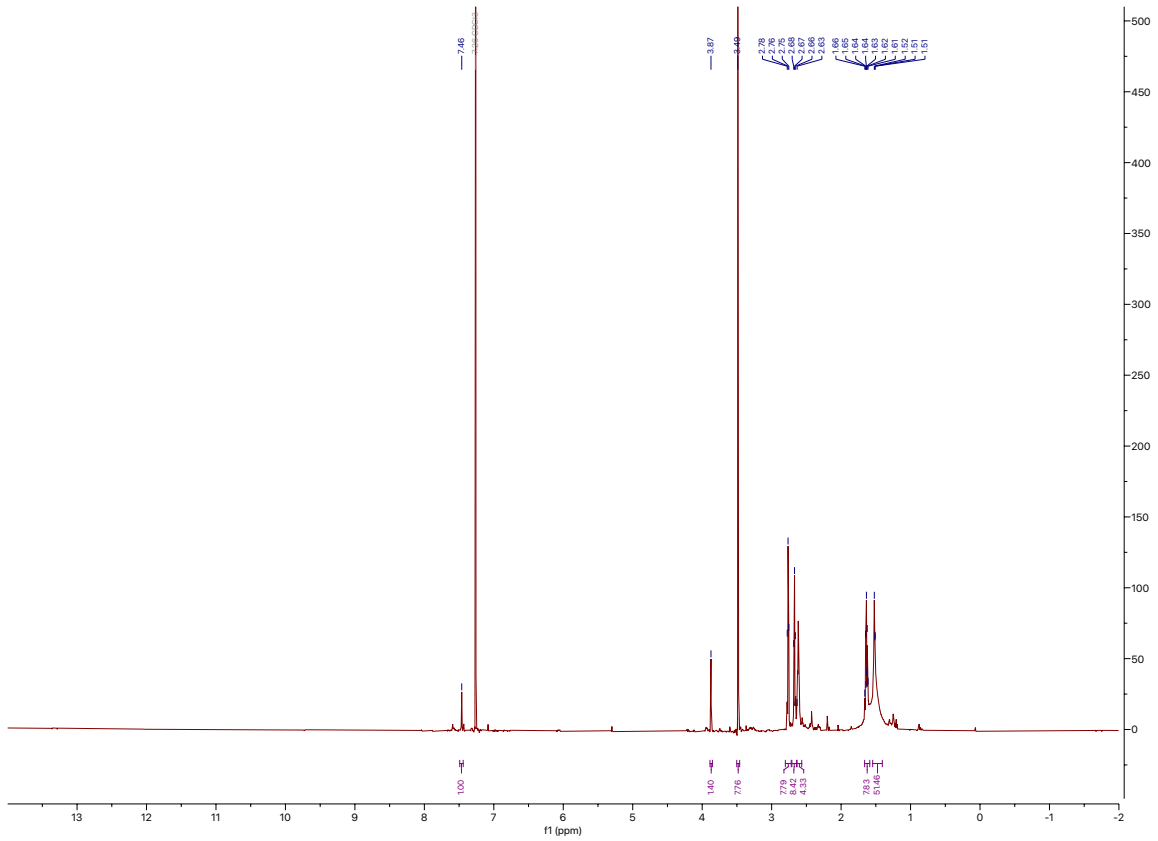


HATU Amide Coupling: In a flame-dried flask under argon was dissolved 3-(3,5-dibromo-4-methoxyphenyl)acrylic acid **(10)** (50.4 mg, 1.0 eq., 0.15 mmol) in 20 mL dichloromethane. To this was added freshly distilled triethylamine (50. µL, 2.4 eq., 0.36 mmol), hexafluorophosphate azabenzotriazole tetramethyl uronium (62.7 mg, 1.10 eq., 0.165 mmol), and spermine (60.1 mg, 1.98 eq., 0.297 mmol). The reaction was stirred at room temperature for 19 hours, then concentrated *in vacuo*. Normal phase column chromatography with a gradient of dichloromethane and 10% ammonium hydroxide in methanol was performed to obtain pure Ianthelliformisamine C **(2)** (12.5 mg, 20%).

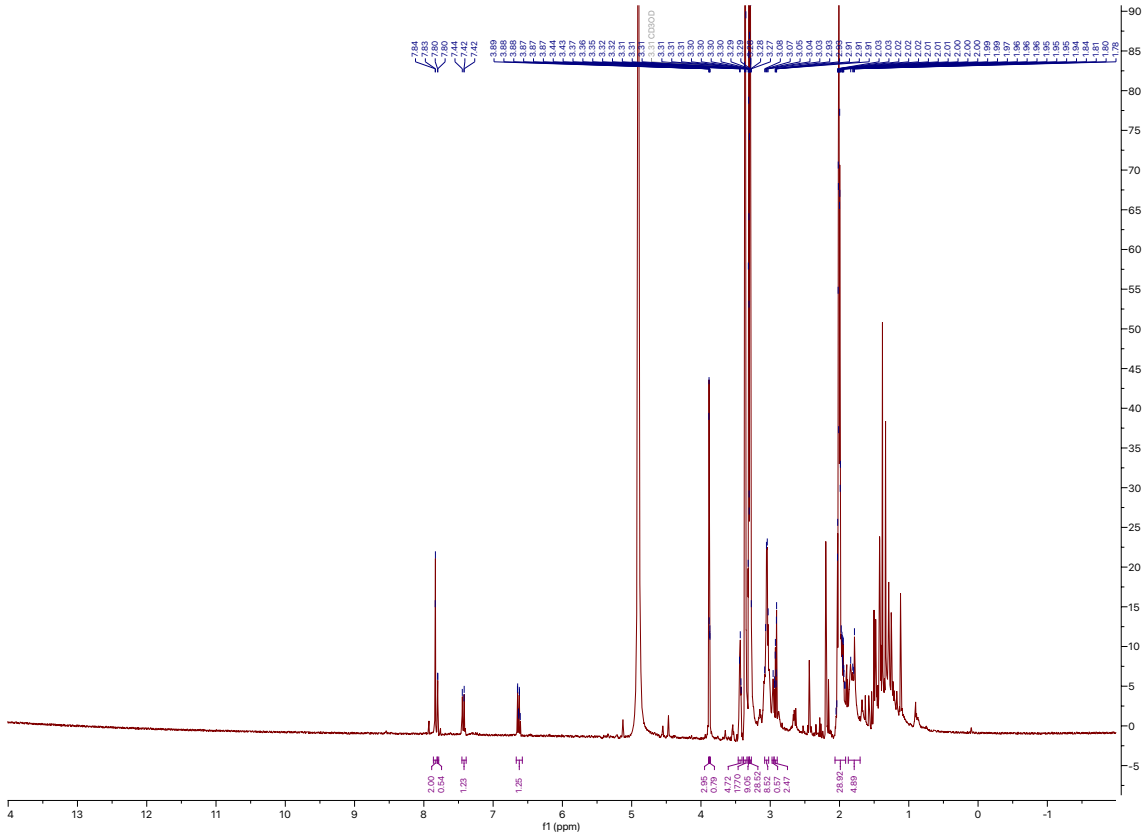
Amidation



In a flame-dried vial under argon was dissolved ethyl (E)-3-(3,5-dibromo-4-methoxyphenyl)acrylate **(9)** (109.2 mg, 0.5916 eq., 0.300 mmol) and spermine (102.6 mg, 1.000 eq., 0.507 mmol) in 4 mL tetrahydrofuran. The reaction was stirred at 64 ºC for 24 hours, then more spermine (106.0 mg, 1.033 eq., 0.5238 mmol) was added, and the reaction was stirred at 64 ºC for another 22 hours. The crude product was concentrated *in vacuo*. A crude NMR showed a possible Michael addition product.



Amidation with TBD: In a flame-dried flask under argon was dissolved ethyl (E)-3-(3,5-dibromo-4-methoxyphenyl)acrylate **(9)** (49.6 mg, 1.00 eq., 0.136 mmol), spermine (59.2 mg, 2.15 eq., 0.293 mmol), and triazabicyclodecene (34.0 mg, 1.79 eq., 0.244 mmol) in 1 mL dichloromethane. The reaction was stirred at 38 ºC for 15 hours, then another 2 mL of dichloromethane was added to replace solvent that had evaporated. The reaction was stirred for another hour, then concentrated *in vacuo*. Normal phase column chromatography was performed with a gradient of methanol with 10% ammonium hydroxide and dichloromethane, and a fraction with a mixture of ethyl 3-(3,5-dibromo-4-methoxyphenyl)acrylate and Ianthelliformisamine C **(2)** was obtained.



Amide Formation Through an Acyl Chloride Intermediate-Trial 1: In a flame-dried vial under argon was dissolved 3-(3,5-dibromo-4-methoxyphenyl)acrylic acid **(10)** (47.9 mg, 1.00 mg, 0.142 mmol) was dissolved in 5 mL dichloromethane. To this was added freshly distilled triethylamine (40 µL, 2.0 eq., 0.29 mmol) and dimethylaminopyridine (8.5 mg, 0.49 eq., 0.070 mmol), and the reaction was cooled to 0 ºC. Thionyl chloride (20 µL, 1.9 eq., 0.29 mmol) was added, and the reaction was stirred at 0 ºC for 15 minutes, then at room temperature for two hours. To this was added spermine (47.6 mg, 1.54 eq., 0.220 mmol), and the reaction was stirred at room temperature for 18 hours then quenched with10 mL of 2 M sodium hydroxide. The quenched mixture was stirred for 10 minutes, then were extracted with ethyl acetate. The organic layer was washed with 2 M sodium hydroxide and extracted another four times with ethyl acetate. The combined organic layers were dried over sodium sulfate, filtered, and evaporated under reduced pressure. A normal phase column was performed with a gradient of dichloromethane and 10% ammonium hydroxide in methanol to isolate protonated ianthelliformisamine C **(11)** (16.7 mg, 27%).

^{1}H NMR (500 MHz, DMSO) δ 8.27 (s, 2H), 7.87 (s, 3H), 7.35 (d, J=16 Hz, 2H), 6.67 (d, J=15 Hz, 2H), 3.81 (s, 6H), 3.25 (m, 4H), 2.77 (m, 9H), 1.73 (m, 4H), 1.61 (m, 4H).

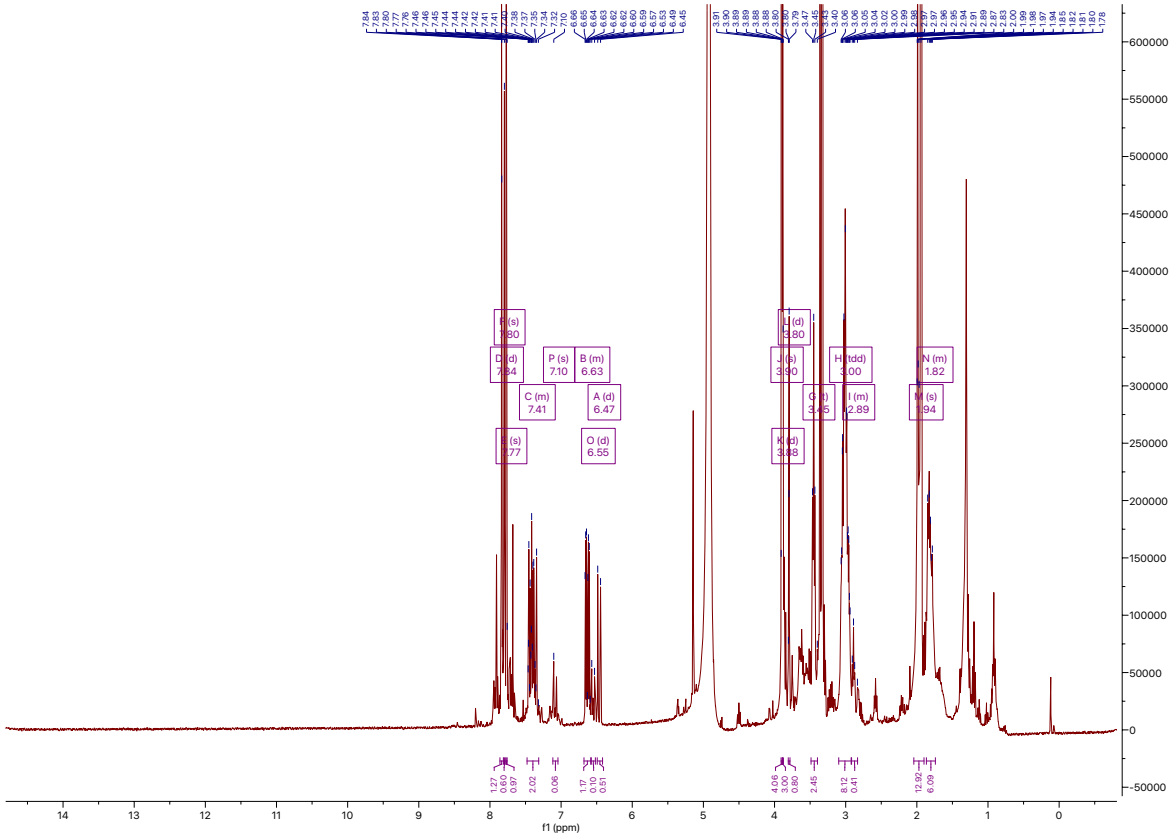
Amide Formation Through an Acyl Chloride Intermediate-Trial 2: In a flame-dried vial under argon was dissolved 3-(3,5-dibromo-4-methoxyphenyl)acrylic acid **(10)** (66.0 mg, 1.00 mg, 0.196 mmol) in 7 mL dichloromethane. To this was added freshly distilled triethylamine (55 µL, 2.0 eq., 0.39 mmol), dimethylaminopyridine (13.2 mg, 0.550 eq., 0.108 mmol), and thionyl chloride (30 µL, 2.1 eq., 0.41 mmol), and the reaction was stirred at room temperature for 90 minutes. Spermine (24.6 mg, 0.619 eq., 0.122 mmol) was added, and the reaction was stirred at room temperature for two days. The reaction was quenched with 10 mL of 1 M sodium hydroxide then extracted four times with ethyl acetate. The combined organic layers were dried over magnesium sulfate, filtered,

and concentrated *in vacuo*. A normal phase column was performed with a gradient of methanol with 10% ammonium hydroxide and DCM to isolate Ianthelliformisamine C **(2)** (6.6 mg, 8%).

Amide Formation Through an Acyl Chloride Intermediate-Trial 3: In a flame-dried flask under argon was dissolved 3-(3,5-dibromo-4-methoxyphenyl)acrylic acid **(10)** (49.1 mg, 1.00 mg, 0.146 mmol) in 5 mL dichloromethane. To this was added freshly distilled triethylamine (40 µL, 2.0 eq., 0.29 mmol), dimethylaminopyrridine (DMAP) (10.1 mg, 0.566 eq., 0.0827 mmol), and thionyl chloride (20 µL, 1.9 eq., 0.28 mmol), and the reaction was stirred at room temperature for two hours. Spermine (149.6 mg, 5.059 eq., 739.3 mmol) was added, and the reaction was stirred at room temperature for 20 hours. The reaction was quenched with 10 mL of 1 M sodium hydroxide then extracted five times with ethyl acetate. The combined organic layers were dried over magnesium sulfate, filtered, and concentrated *in vacuo*. A normal phase column was performed with a gradient of dichloromethane and methanol with 10% ammonium hydroxide to isolate Ianthelliformisamine C (3.7 mg, 6%).

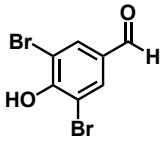
Amide Formation Through an Acyl Chloride Intermediate with Acidic Work Up and Extraction: In a flame-dried flask under argon was dissolved 3-(3,5-dibromo-4-methoxyphenyl)acrylic acid **(10)** (473.0 mg, 1.00 mg, 1.408 mmol) in 5 mL dichloromethane. To this was added freshly distilled triethylamine (520 µL, 2.65 eq., 3.73 mmol), and the reaction was cooled to 0 ºC. Thionyl chloride (120 µL, 1.17 eq., 1.64 mmol) was added, and the reaction was stirred at 0 ºC for two hours. Spermine (1.6657 g, 5.847 eq., 8.2318 mmol) was dissolved in 7.5 mL of dichloromethane in a flame dried 25 mL flask under argon. The acyl chloride solution was transferred into the spermine solution, and the reaction was stirred for 23 hours starting at 0 ºC and warming to room temperature. The reaction mixture was diluted with ethyl acetate and washed three times with 0.6 M hydrochloric acid. After each wash, the aqueous layer was immediately

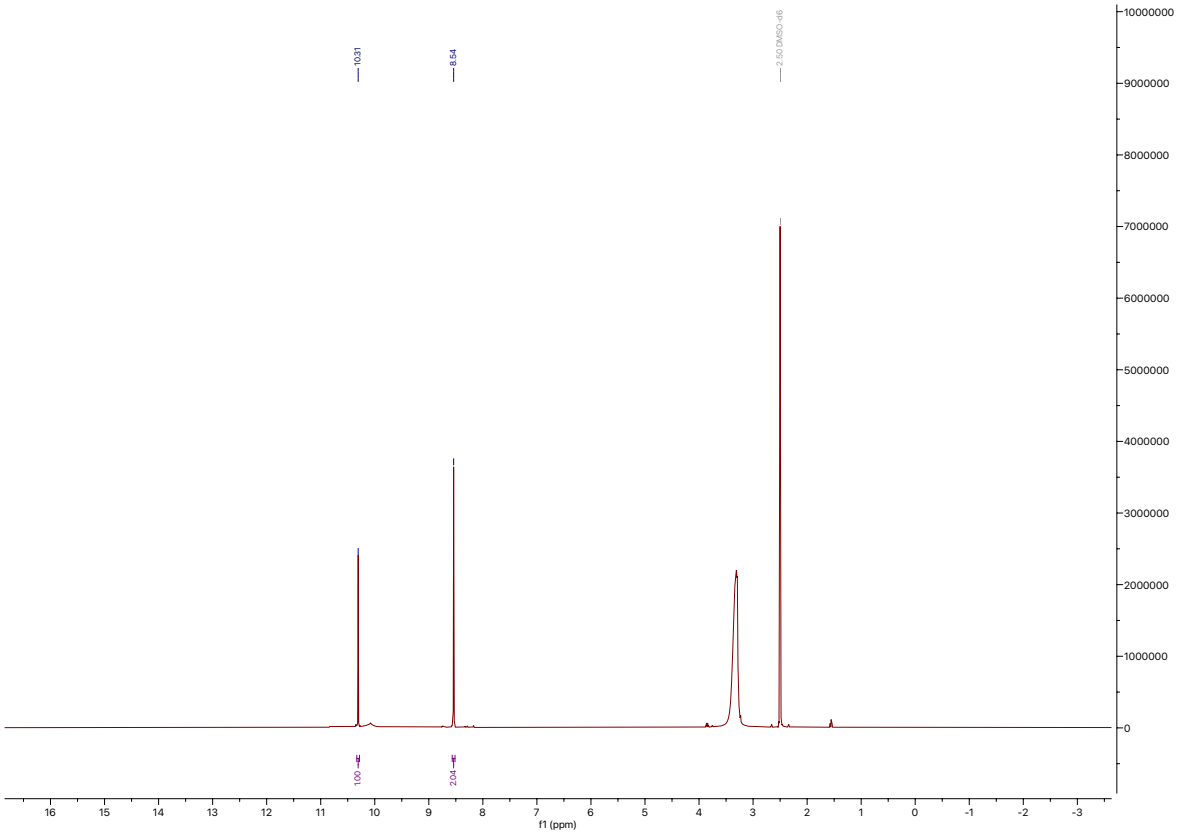
basified to pH 14 with 1 M sodium hydroxide. The combined aqueous layers were extracted three times with ethyl acetate and methanol. The combined organic layers were dried over magnesium sulfate, filtered, and concentrated *in vacuo*. The products were extracted from a crude solid by dissolving them in methanol and leaving behind the undesired byproducts. The methanol solution was concentrated *in vacuo*, and NMR showed a mixture of ianthelliformisamine A and C and other impurities.

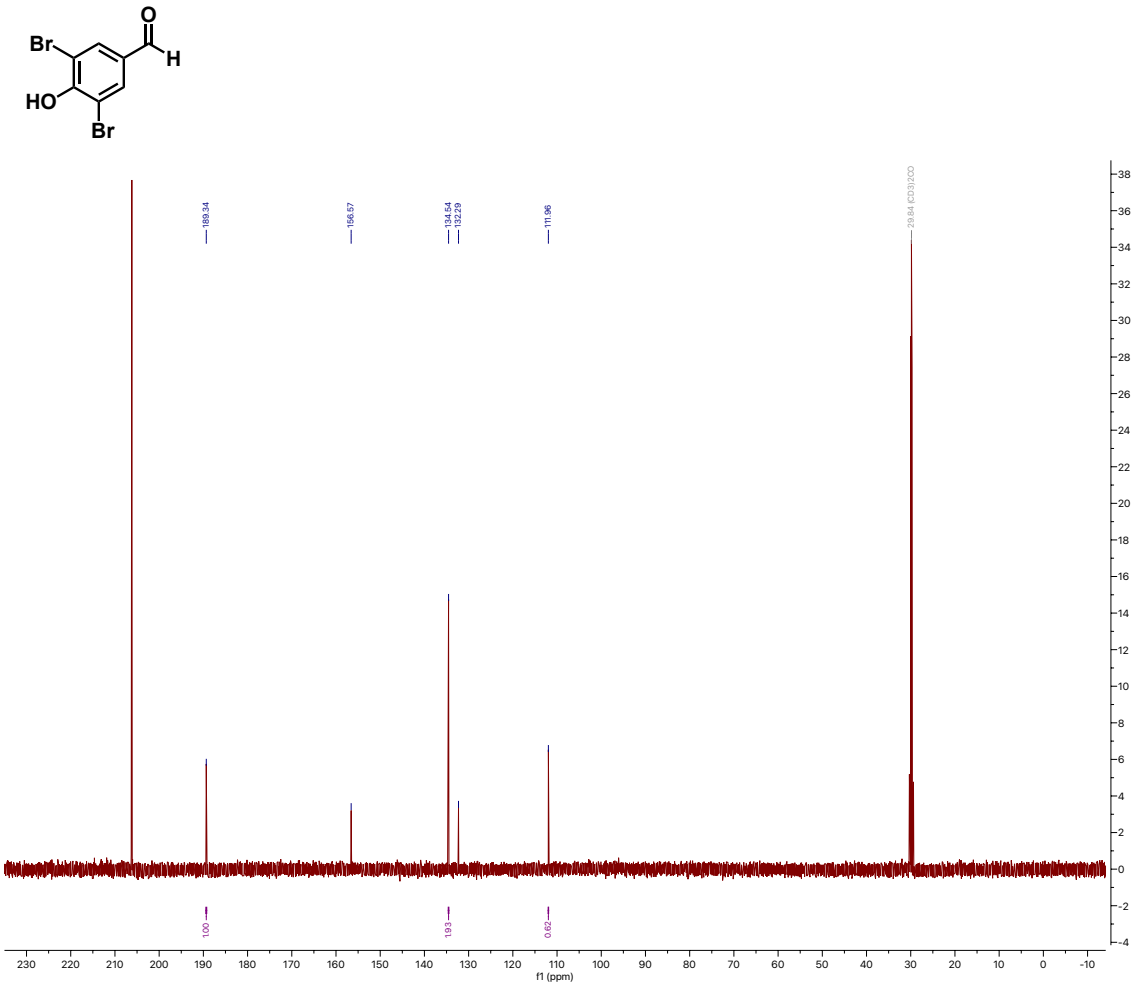


5.4 Spectral Data

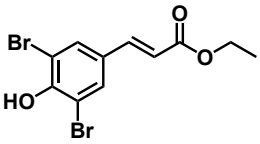
3-5-dibromo-4-hydroxybenzaldehyde (7)

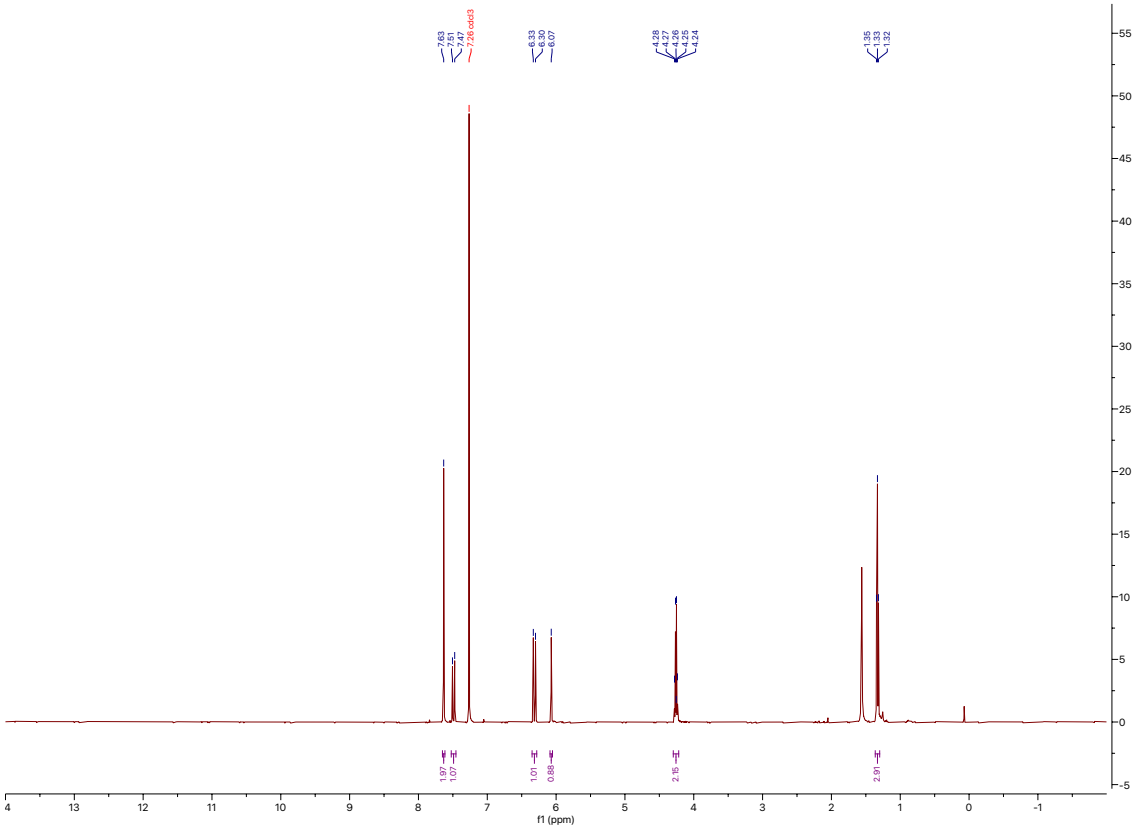


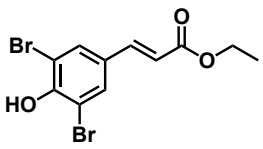


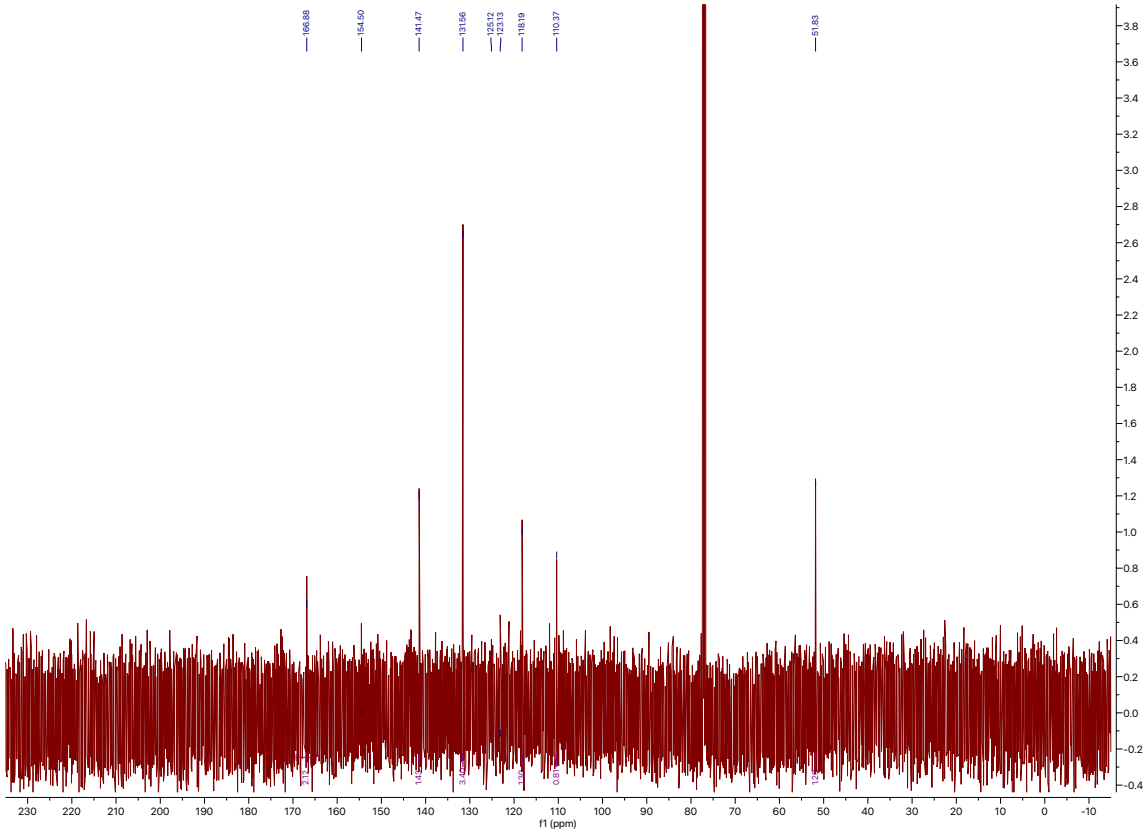

Br
HO
Br
O
H
189.34
156.57
134.54
132.29
111.96
29.84 (CD3)2CO
1.00
1.93
0.62
f1 (ppm)

Ethyl 3-(3,5-dibromo-4-hydroxyphenyl)acrylate (8)

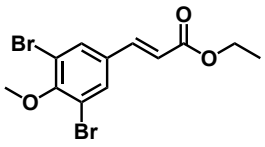


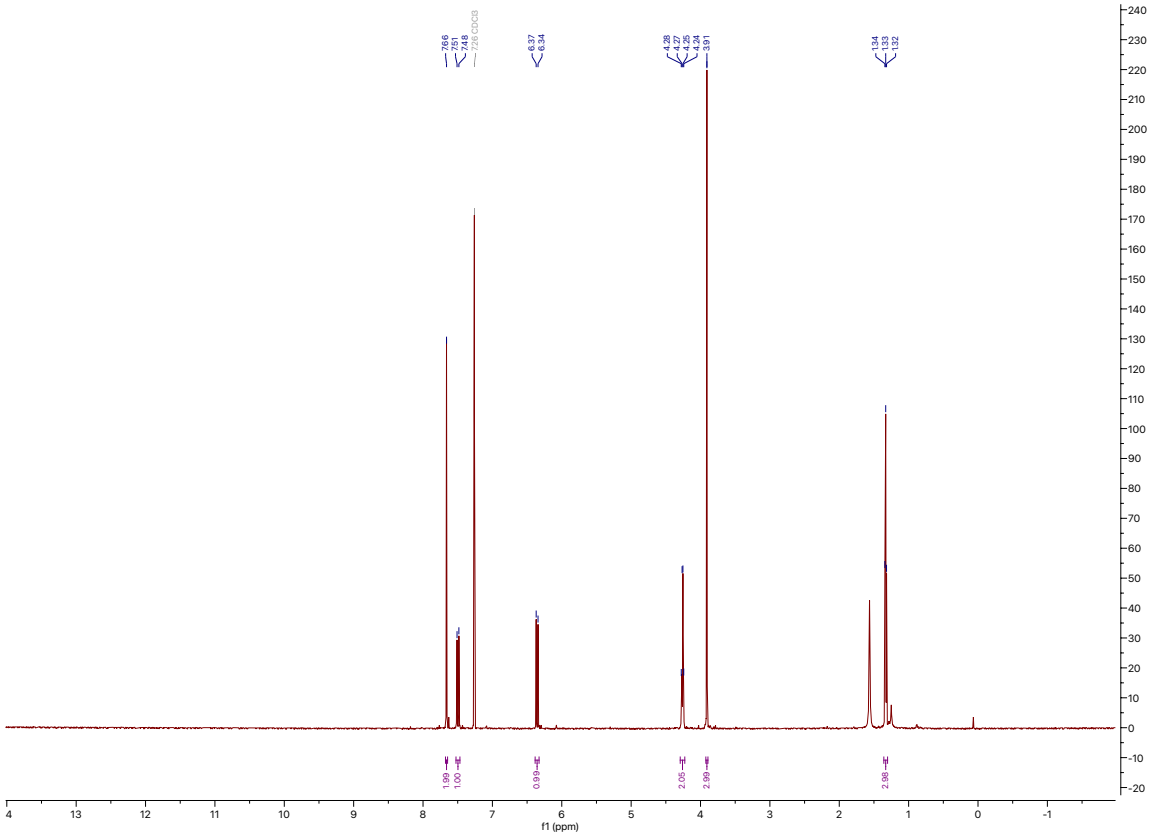


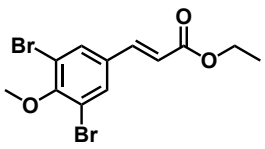

Br
HO
Br
O
O

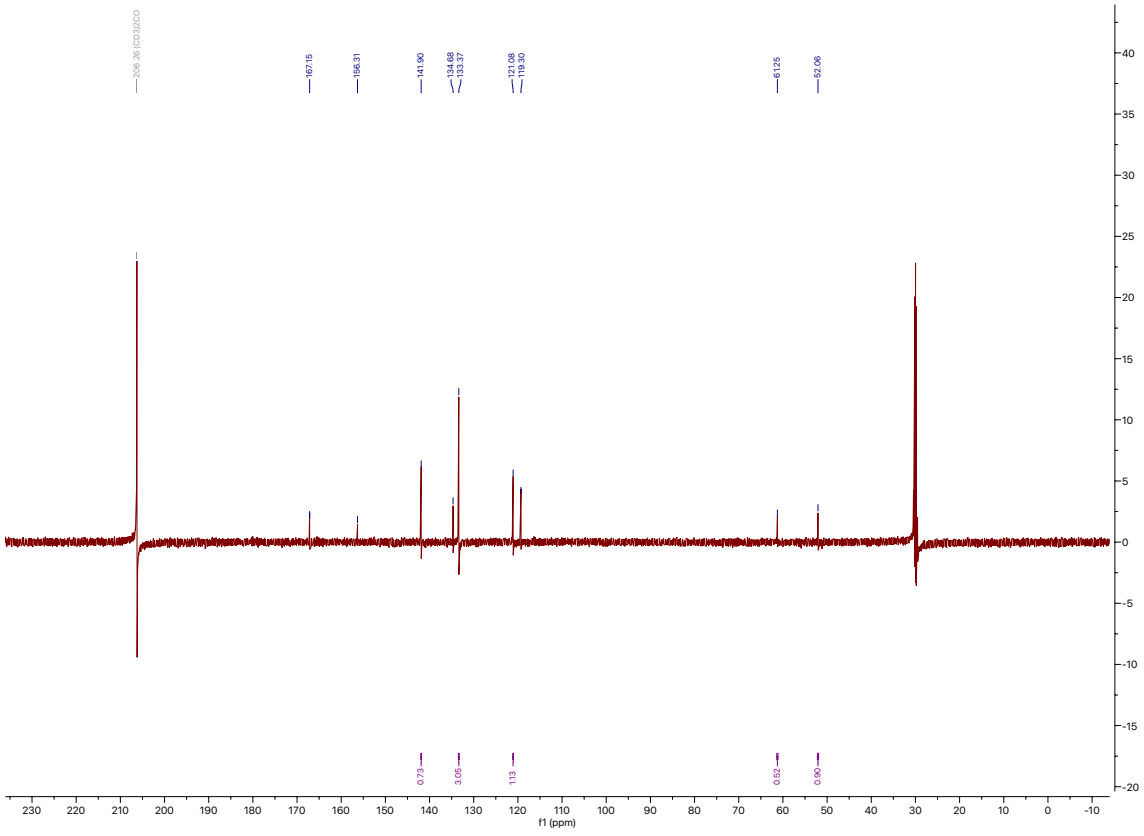

166.88
154.50
141.47
131.56
125.12
123.13
118.19
110.37
51.83
f1 (ppm)

Methyl 3-(3,5-dibromo-4-methoxyphenyl)acrylate (9)

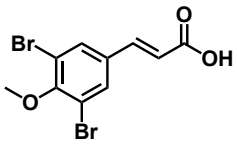


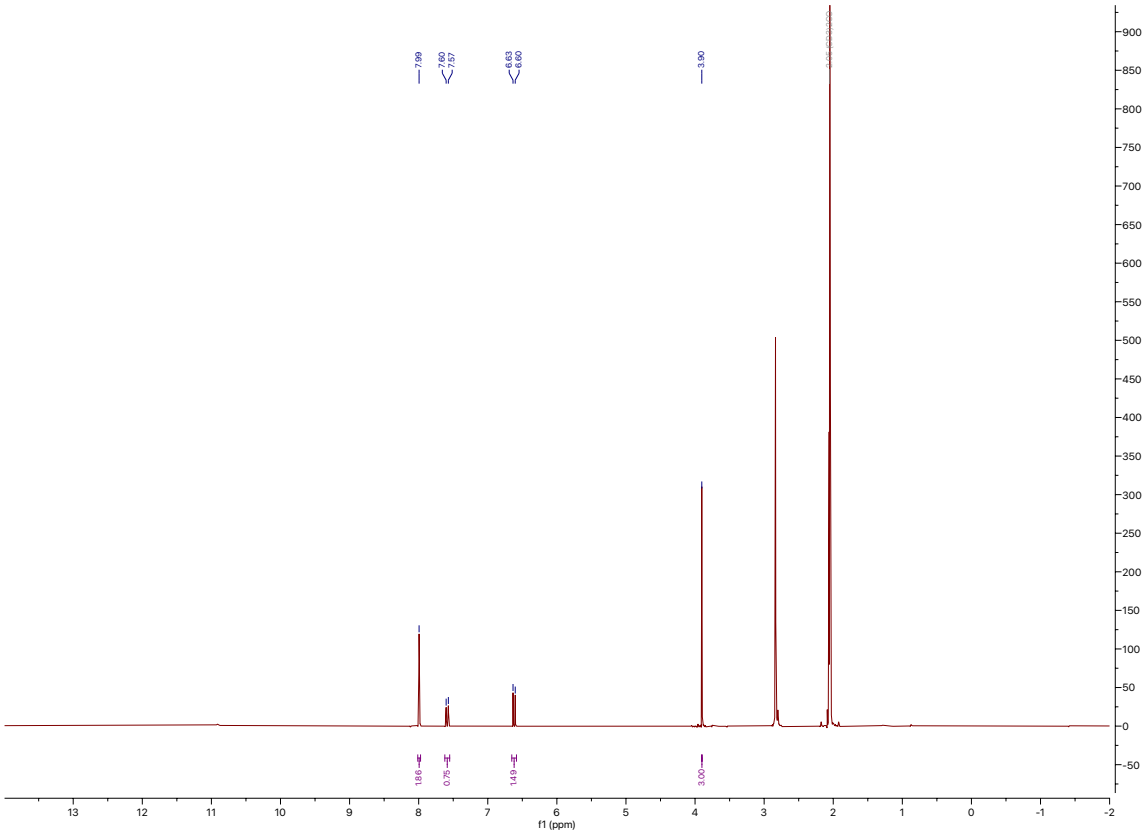



Br
O
O
O
Br

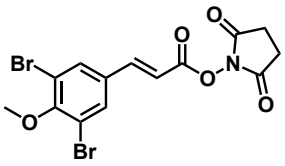

206.26 (CD3)2CO
167.15
156.31
141.90
134.68
133.37
121.08
119.30
61.25
52.06
0.73
3.05
1.13
0.52
0.90
f1 (ppm)

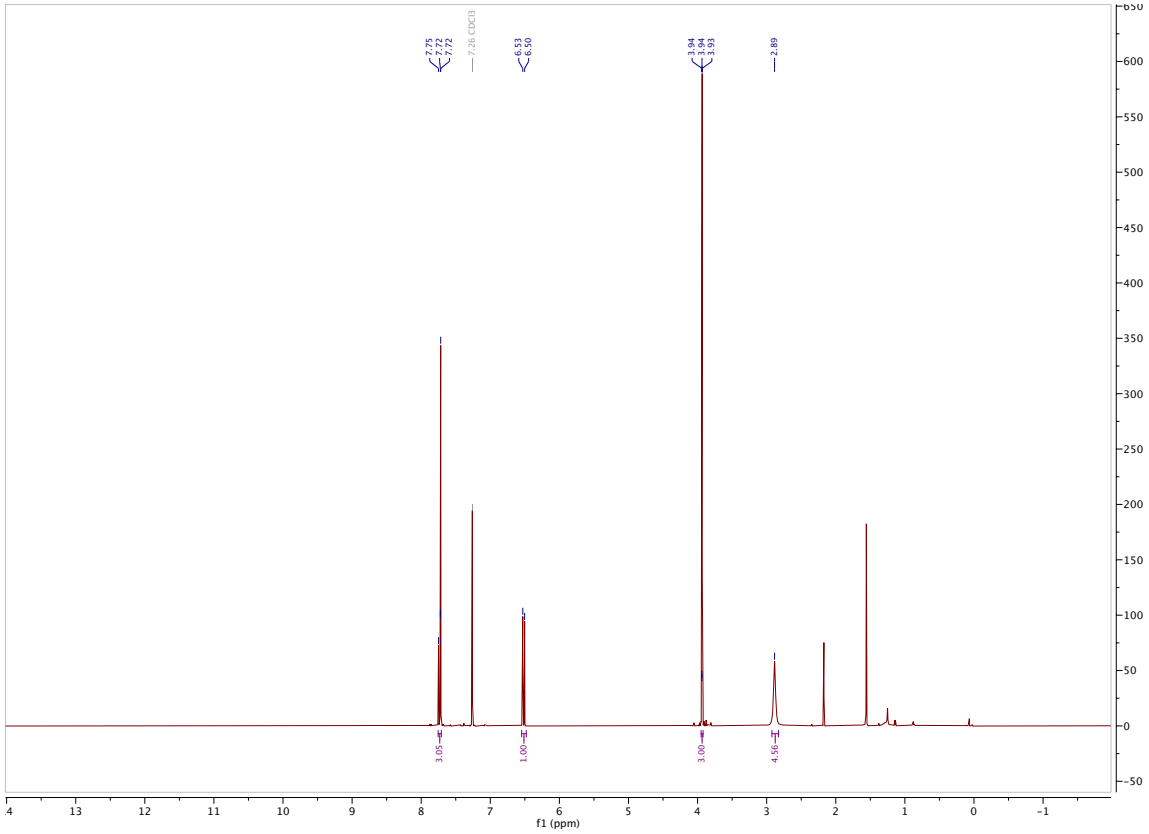
3-(3,5-dibromo-4-methoxyphenyl)acrylic acid (10)

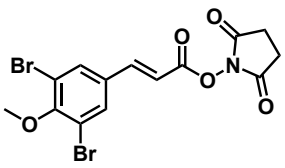


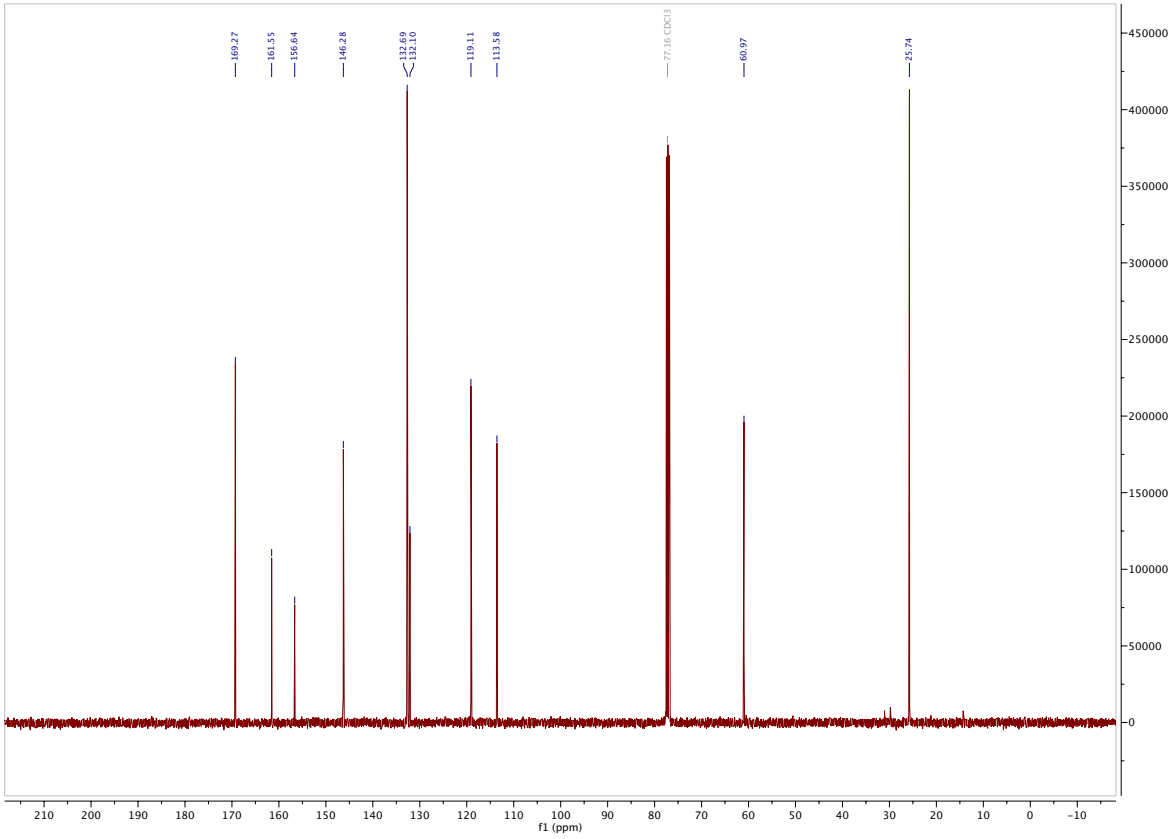


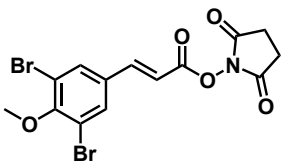
2,5-dioxopyrrolidin-1-yl (E)-3-(3,5-dibromo-4-methoxyphenyl)acrylate

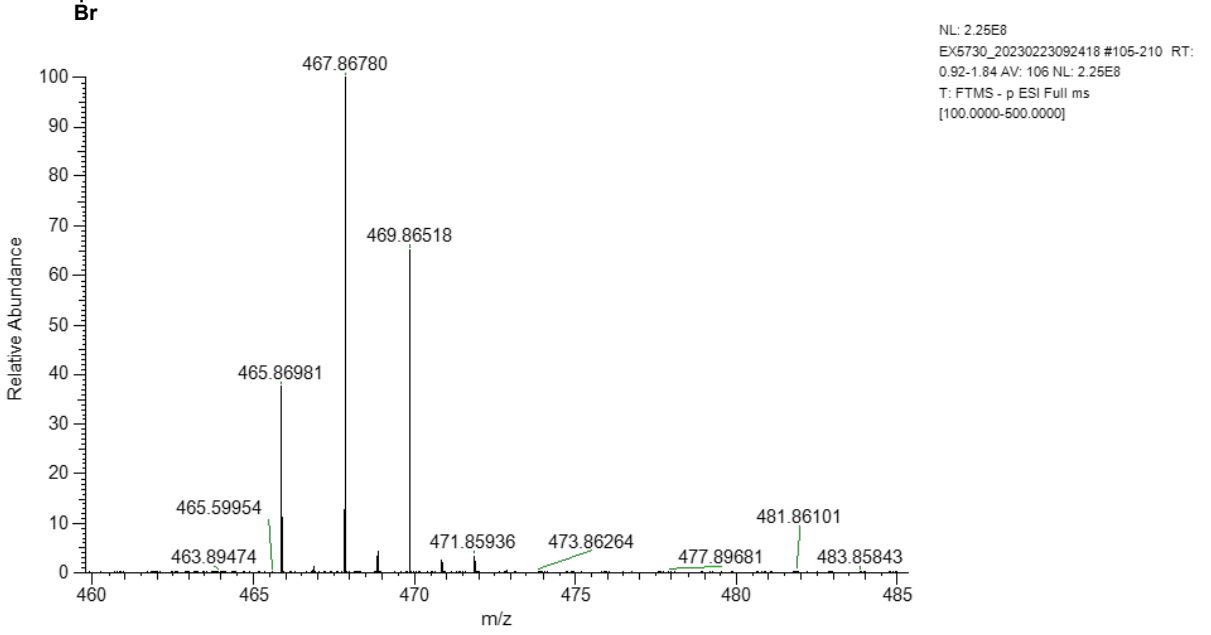


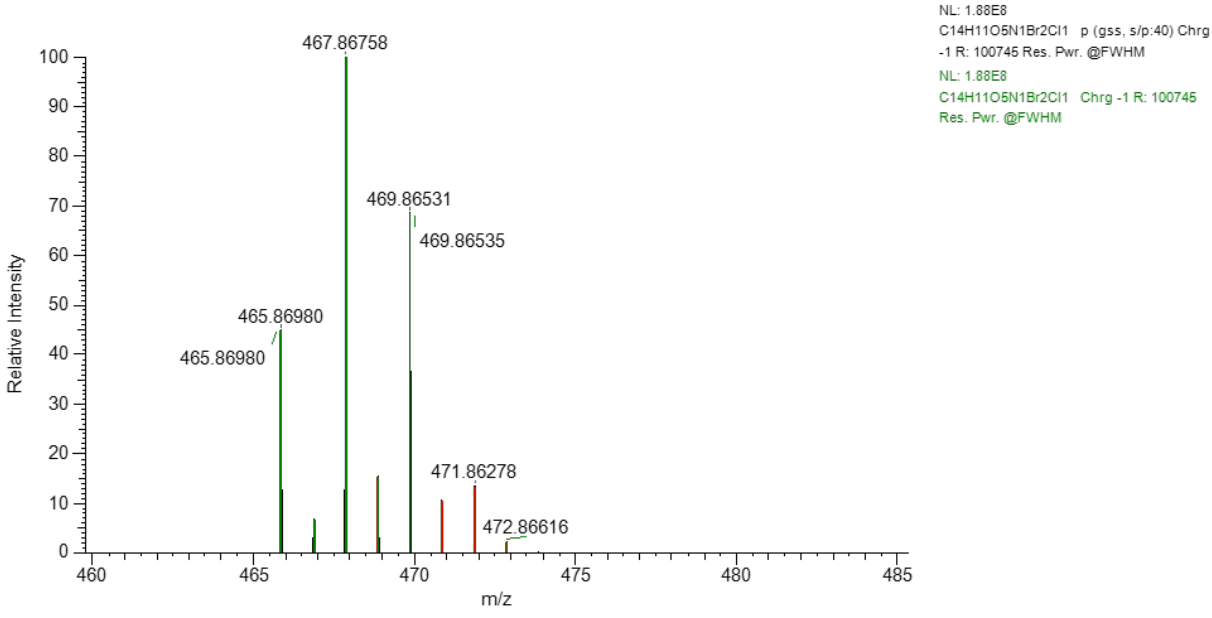



Br
O
O
O
N
O
O
Br

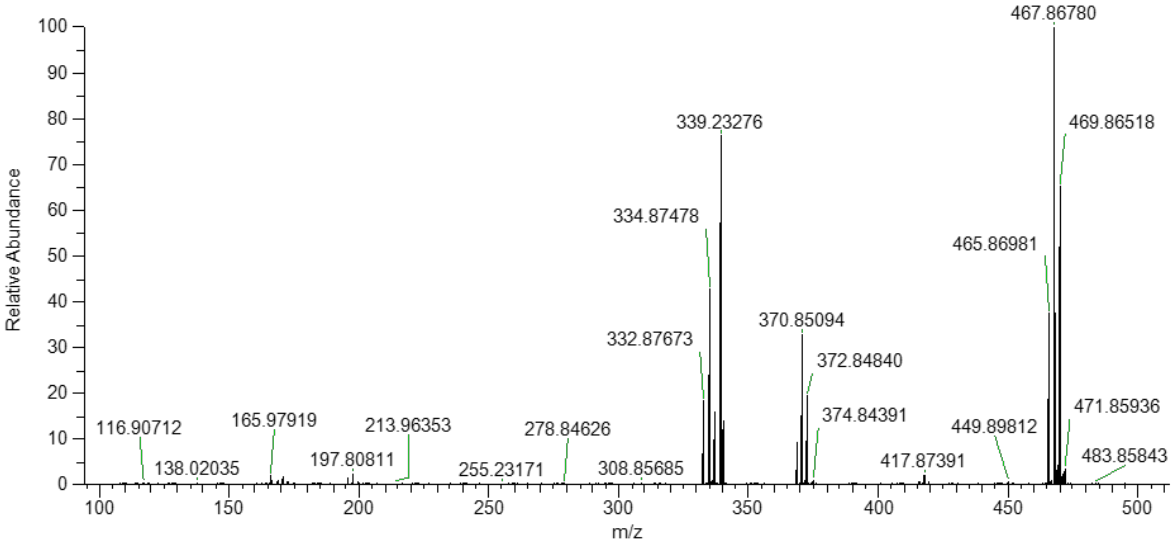

169.27
161.55
156.64
146.28
132.69
132.10
119.11
113.58
77.16 CDCl3
60.97
25.74
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
−10
f1 (ppm)


Br
O
O
O
N
O
O
Br

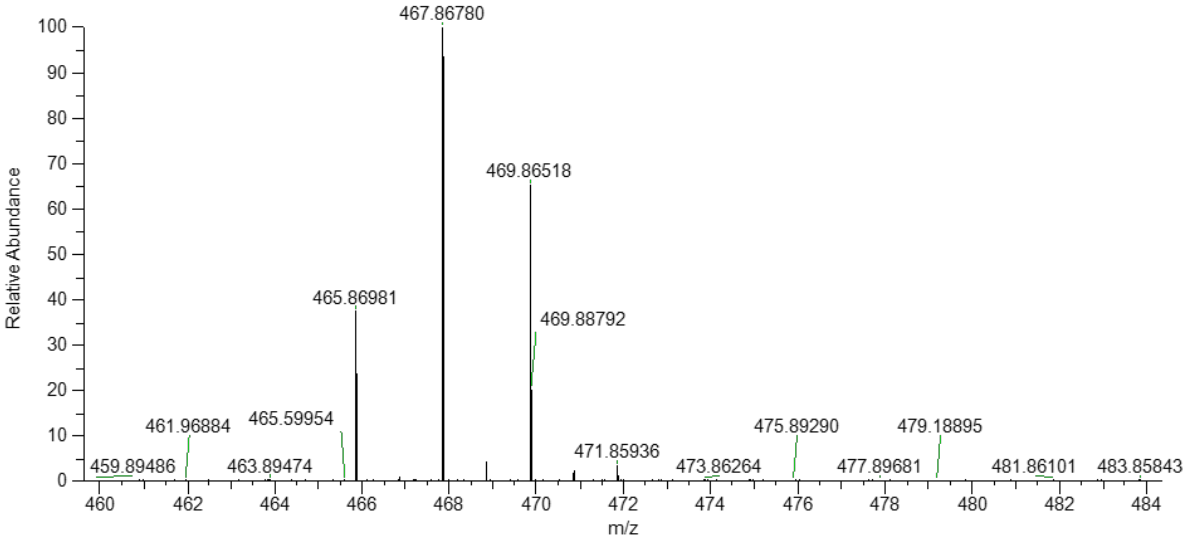

NL: 2.25E8
EX5730_20230223092418 #105-210 RT: 0.92-1.84 AV: 106 NL: 2.25E8
T: FTMS - p ESI Full ms [100.0000-500.0000]
467.86780
469.86518
465.86981
465.59954
463.89474
471.85936
473.86264
477.89681
481.86101
483.85843
Relative Abundance
m/z


NL: 1.88E8
C14H11O5N1Br2Cl1 p (gss, s/p:40) Chrg -1 R: 100745 Res. Pwr. @FWHM
NL: 1.88E8
C14H11O5N1Br2Cl1 Chrg -1 R: 100745 Res. Pwr. @FWHM
467.86758
469.86531
469.86535
465.86980
465.86980
471.86278
472.86616
Relative Intensity
m/z

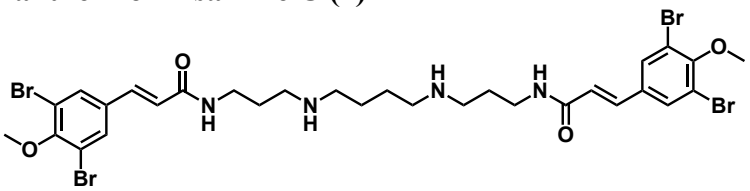
EX5730_20230223092418 #105-210 RT: 0.92-1.84 AV: 106 NL: 2.25E8
T: FTMS - p ESI Full ms [100.0000-500.0000]

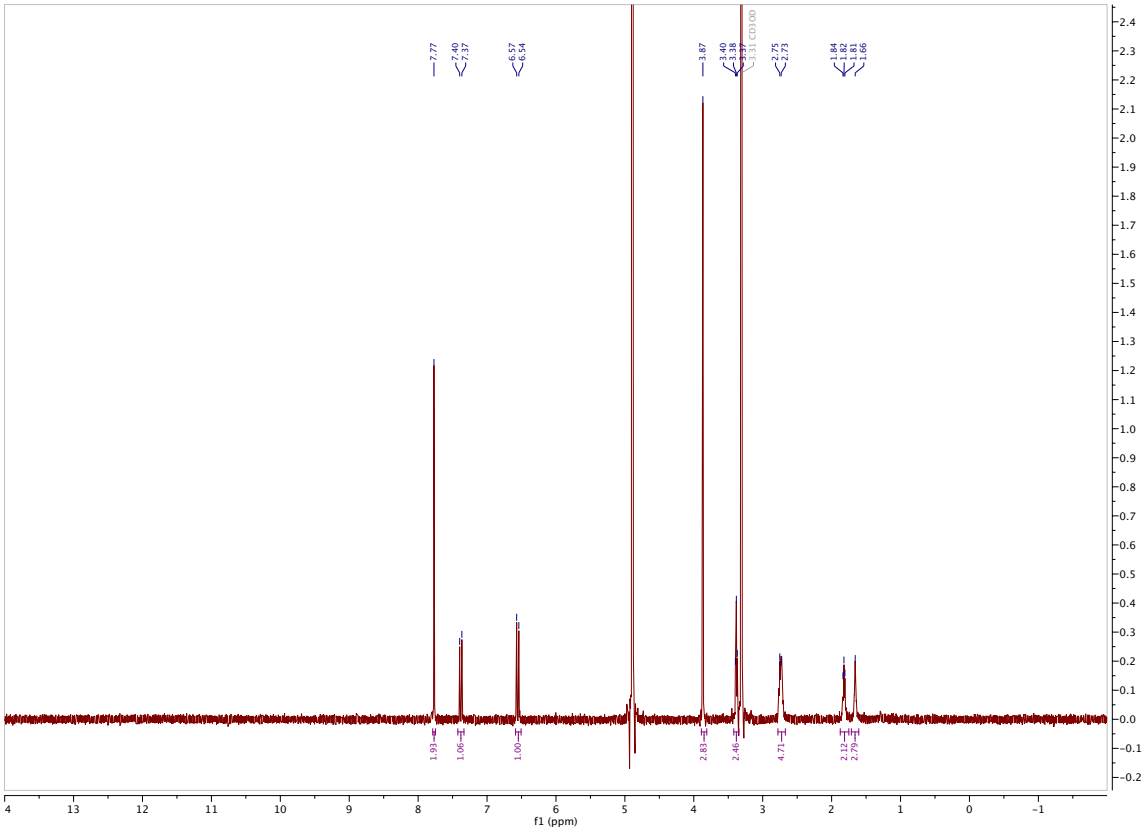


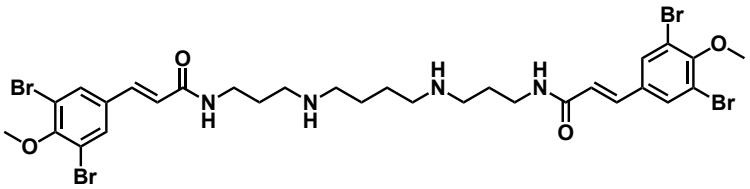
EX5730_20230223092418 #105-210 RT: 0.92-1.84 AV: 106 NL: 2.25E+008
T: FTMS - p ESI Full ms [100.0000-500.0000]

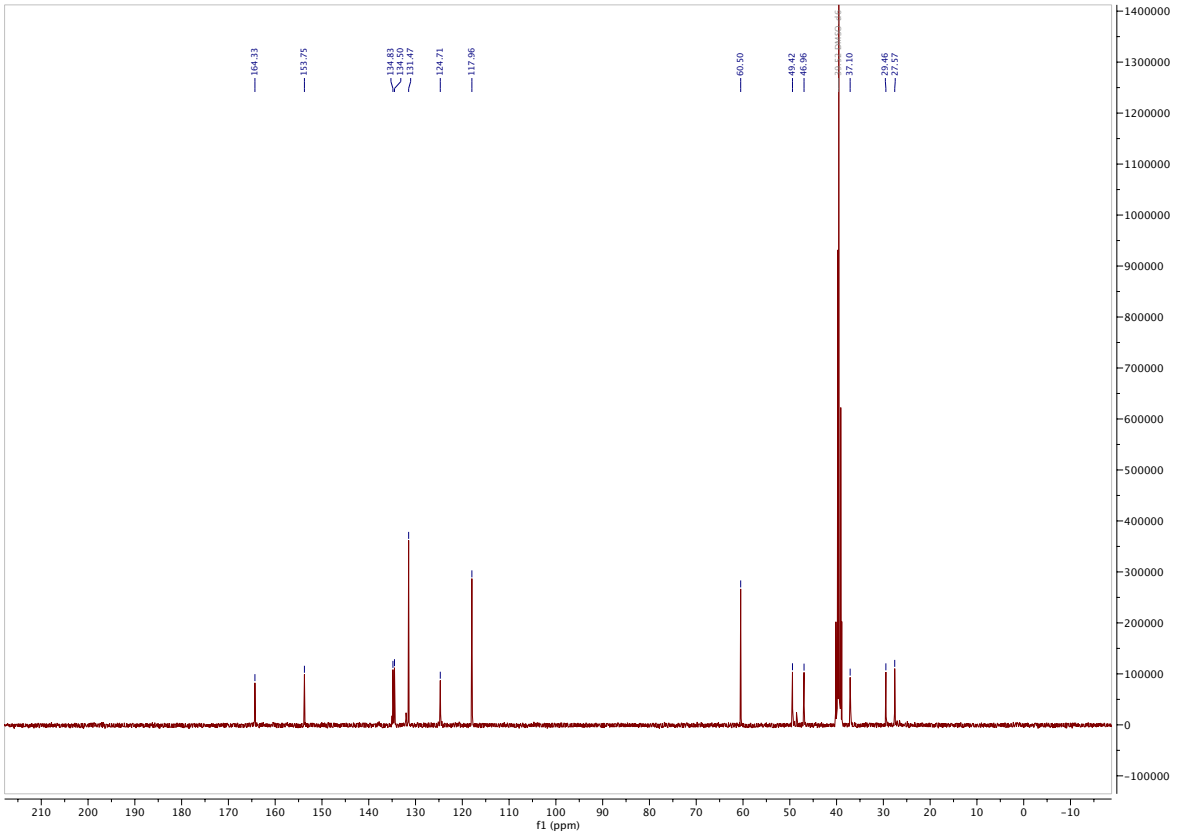


Ianthelliformisamine C (2)

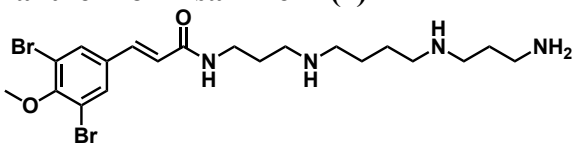


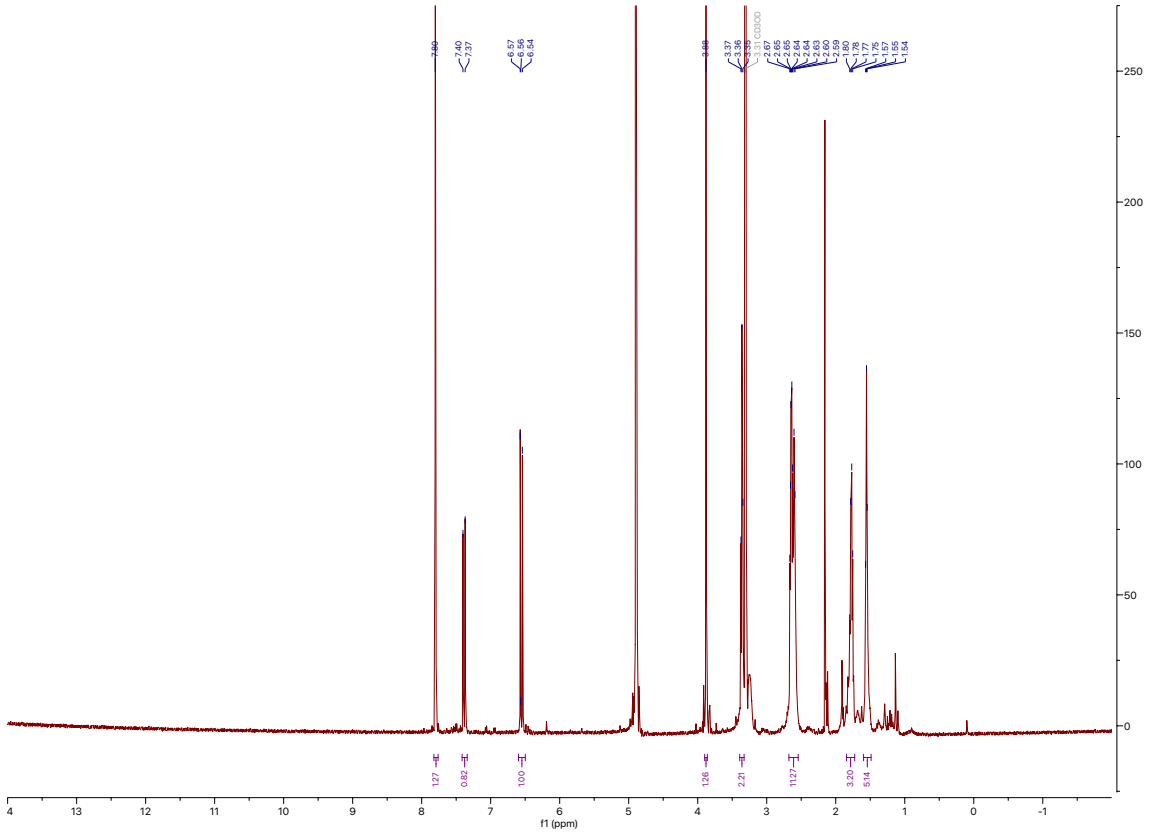



Br
O
N
H
N
H
H
N
H
N
O
Br
O
Br
Br

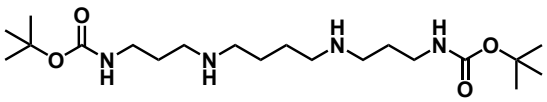

164.33
153.75
134.83
134.50
131.47
124.71
117.96
60.50
49.42
46.96
37.10
29.46
27.57
1400000
1300000
1200000
1100000
1000000
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
-100000
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

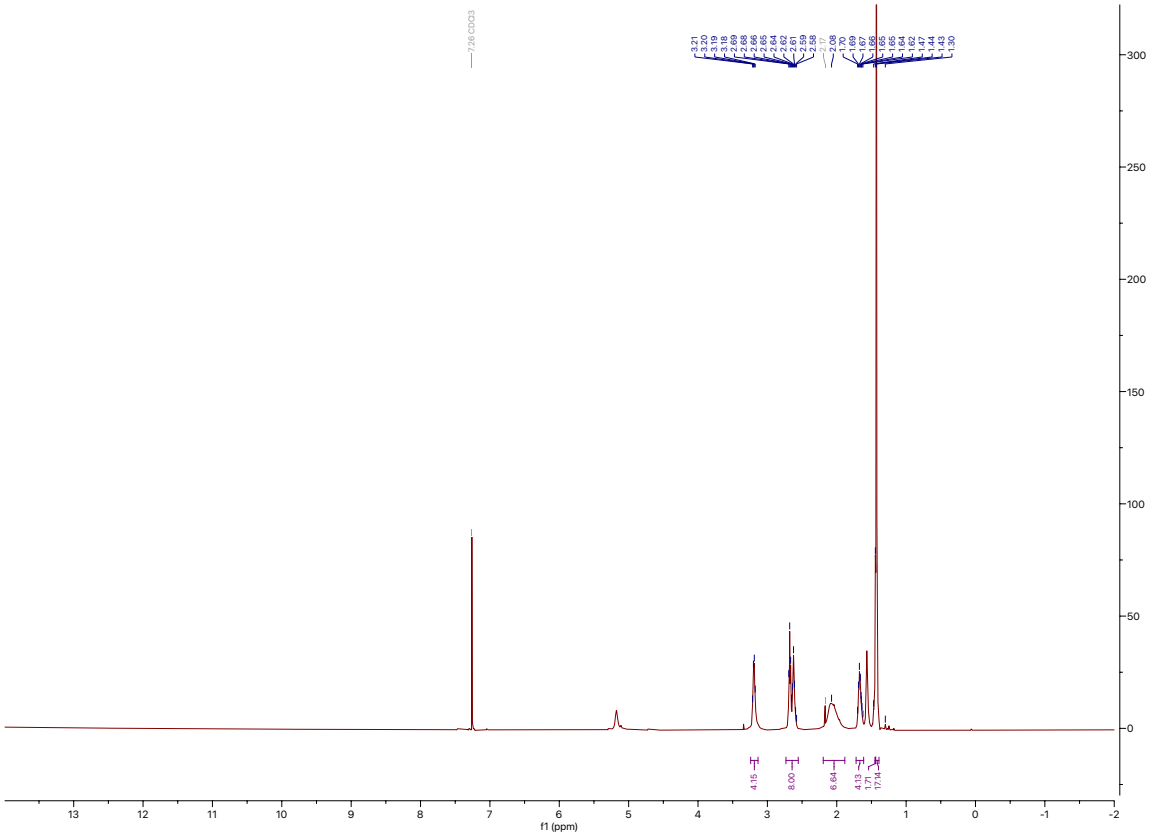
Ianthelliformisamine A (1)



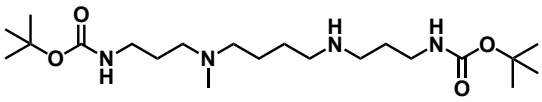


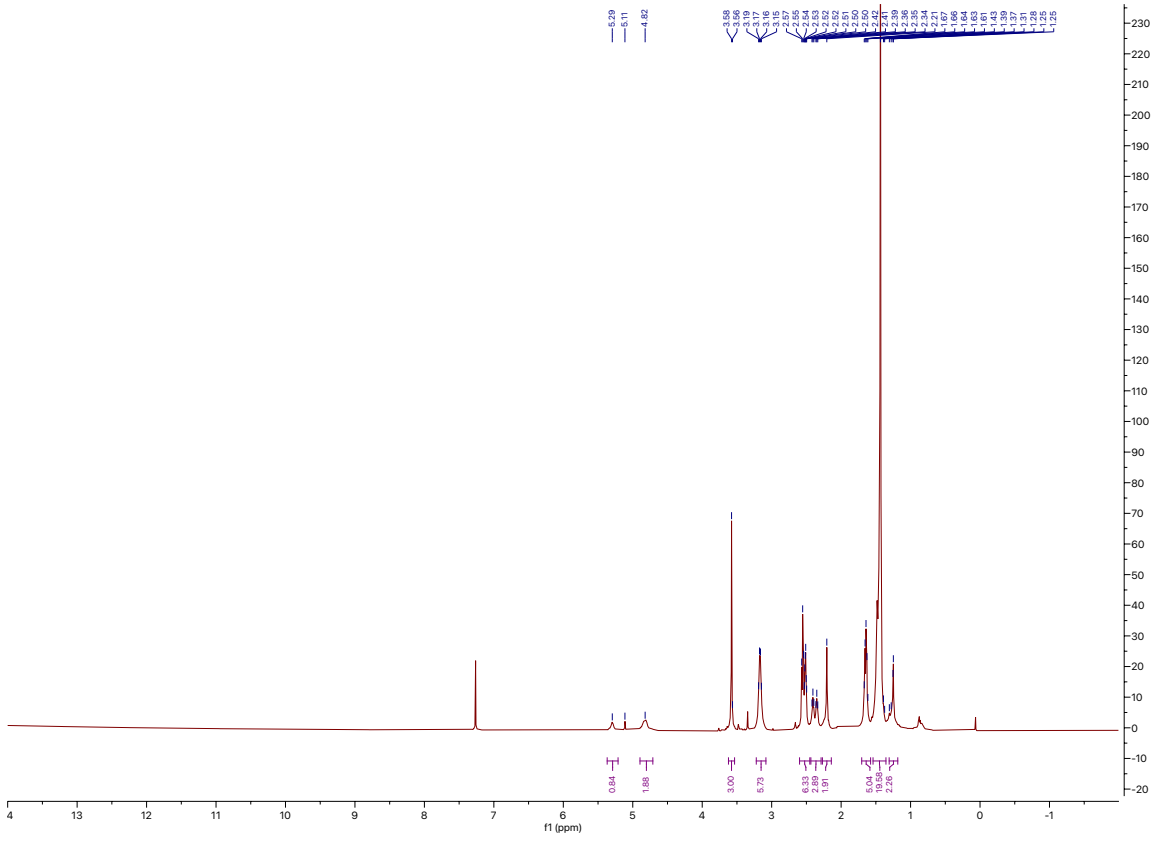
Di-tert-butyl ((butane-1,4-diylbis(azanediyl))bis(propane-3,-diyl))dicarbamate



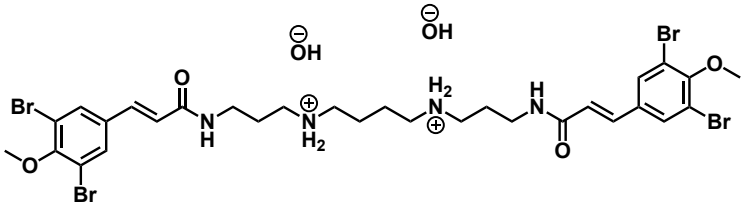


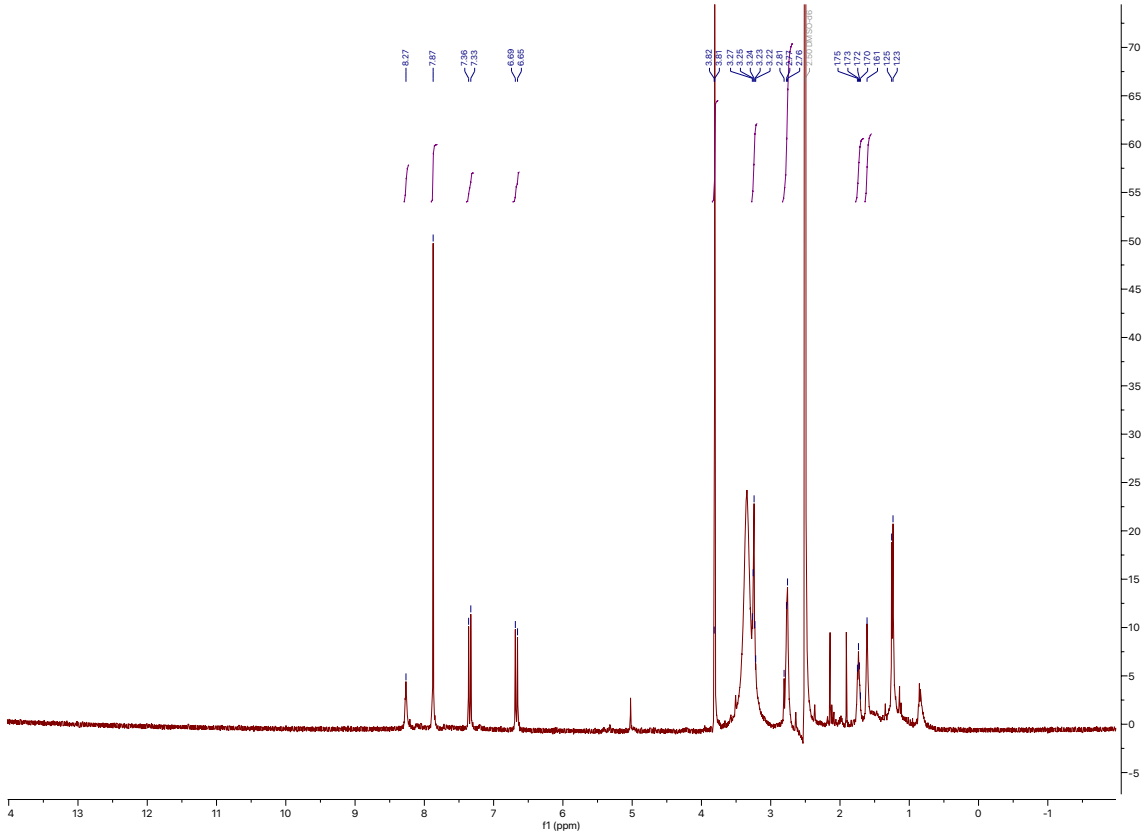
Tert-butyl (2,2,14-trimethyl-4-oxo-3-oxa-5,9,14-triazaheptadecan-17-yl) carbamate



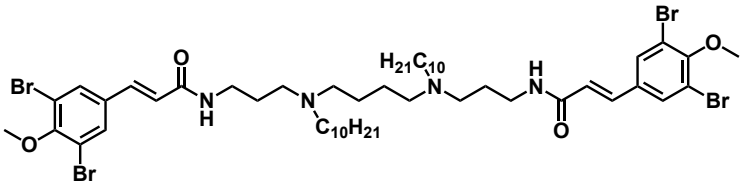


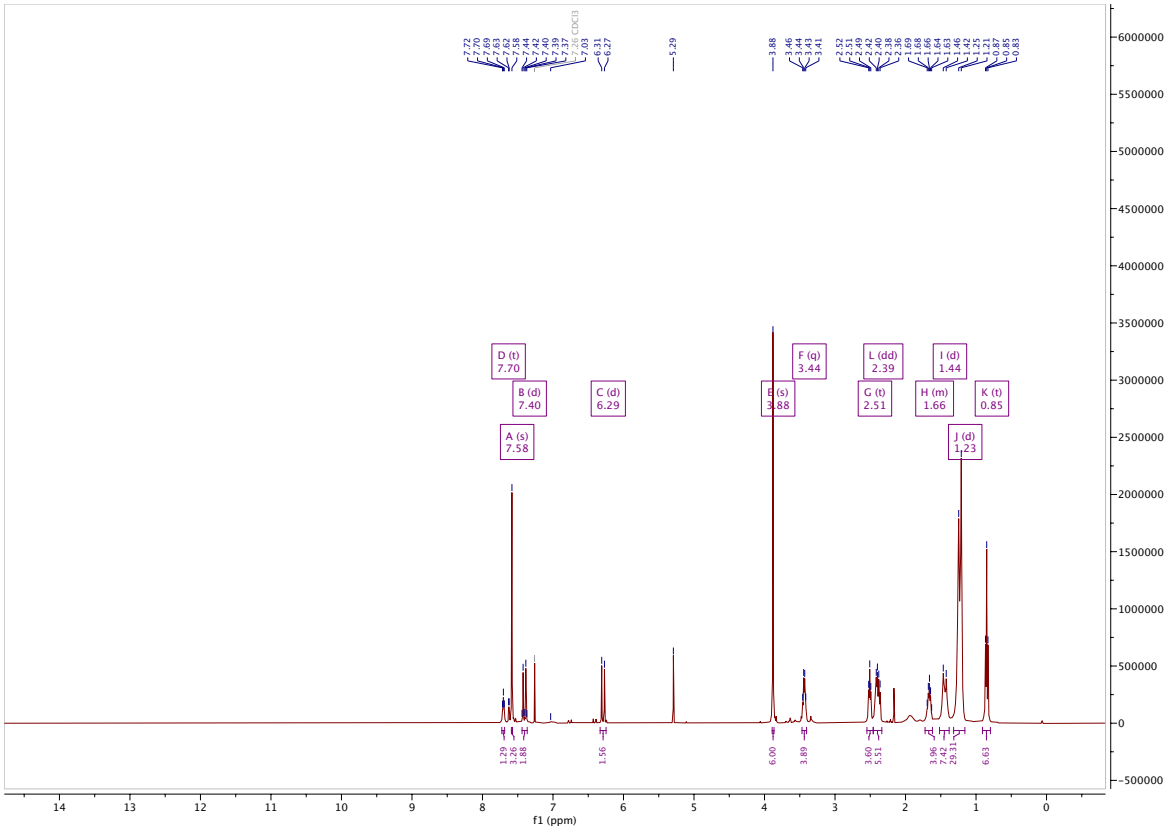
Protonated Ianthelliformisamine C Hydroxide Salt (11)

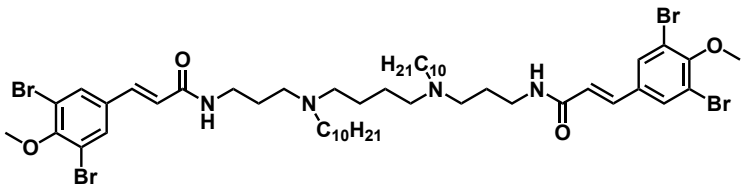


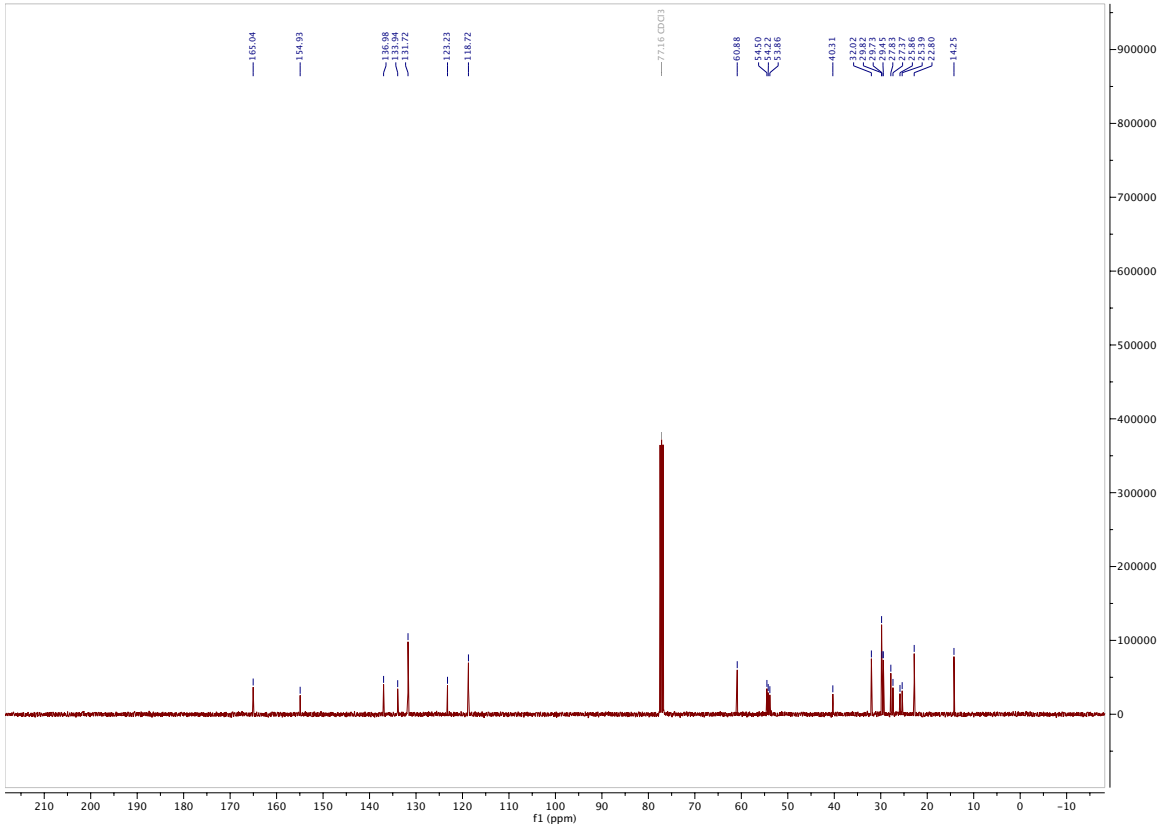


(2E,2'E)-N,N'-((butane-1,4-diylbis(decylazanediyl))bis(propane-3,1-diyl))bis(3-(3,5-dibromo-4-methoxyphenyl)acrylamide)

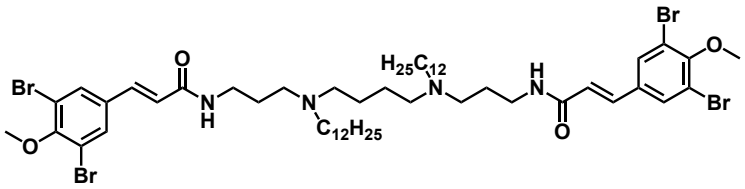


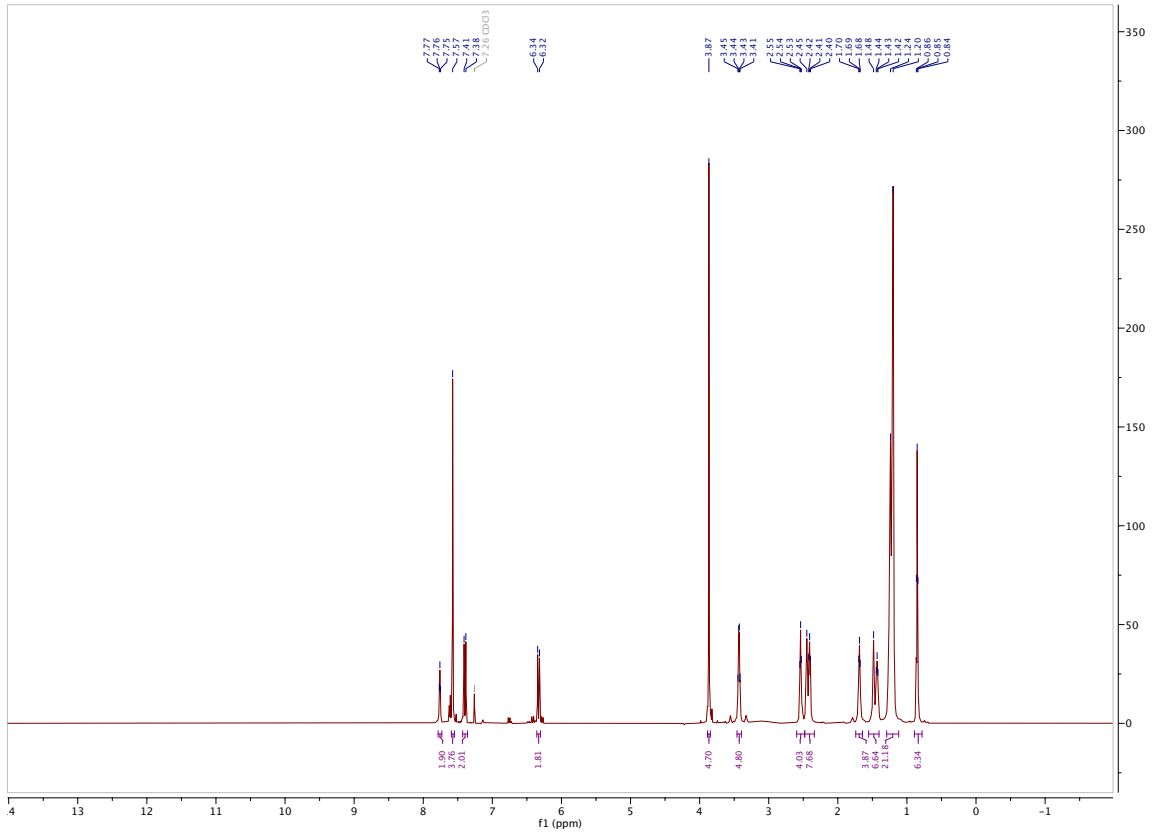


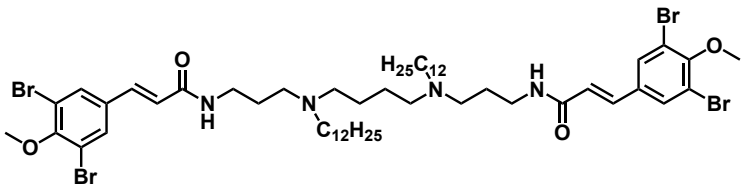

Br
O
N
H
Br
O
$H_{21}C_{10}$
N
N
$C_{10}H_{21}$
H
N
O
Br
O
Br

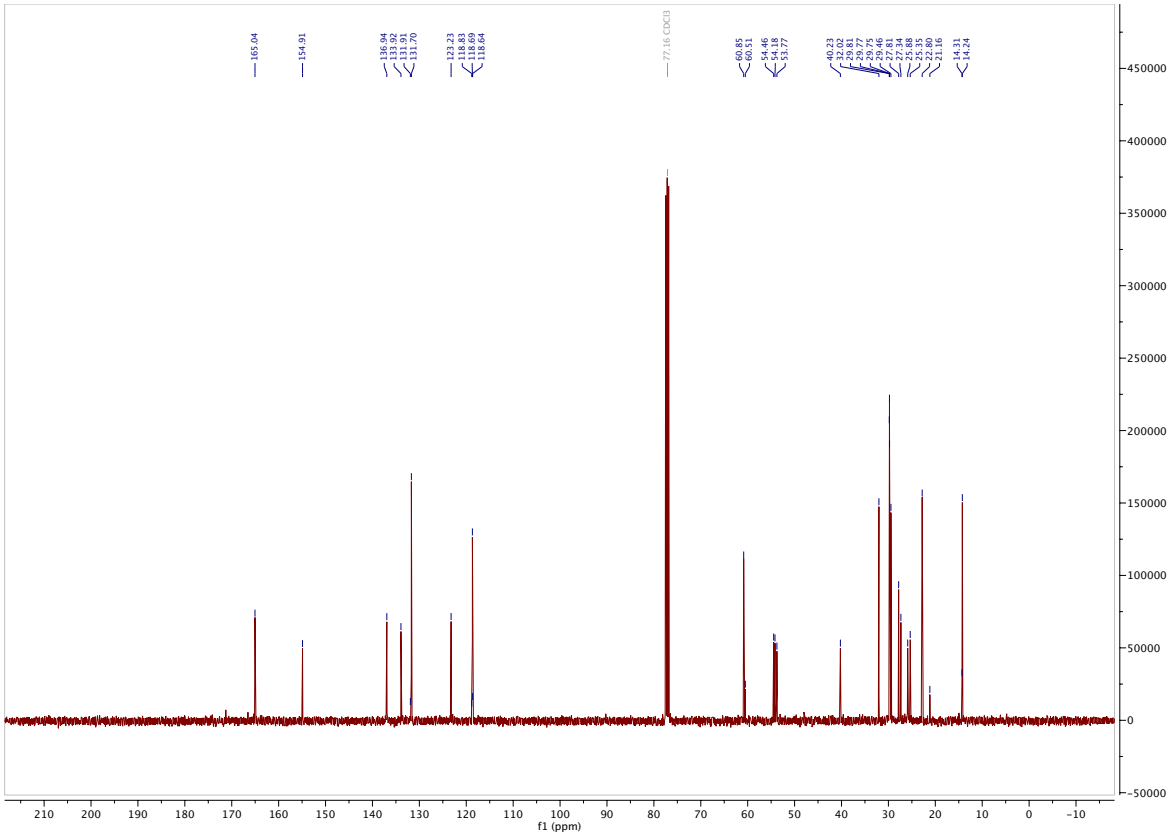

165.04
154.93
136.98
131.84
131.72
123.23
118.72
77.16 CDCl3
60.88
54.50
54.22
53.86
40.31
32.02
29.82
29.73
29.54
27.83
27.37
26.66
23.39
22.80
14.25
900000
800000
700000
600000
500000
400000
300000
200000
100000
0
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

(2E,2'E)-N,N'-((butane-1,4-diylbis(dodecylazanediyl))bis(propane-3,1-diyl))bis(3-(3,5-dibromo-4-methoxyphenyl)acrylamide)

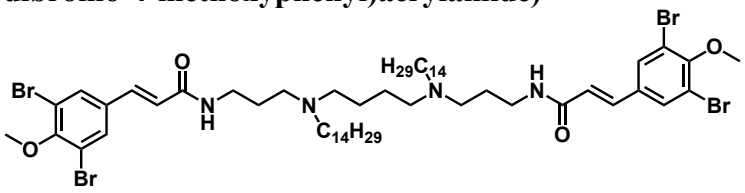


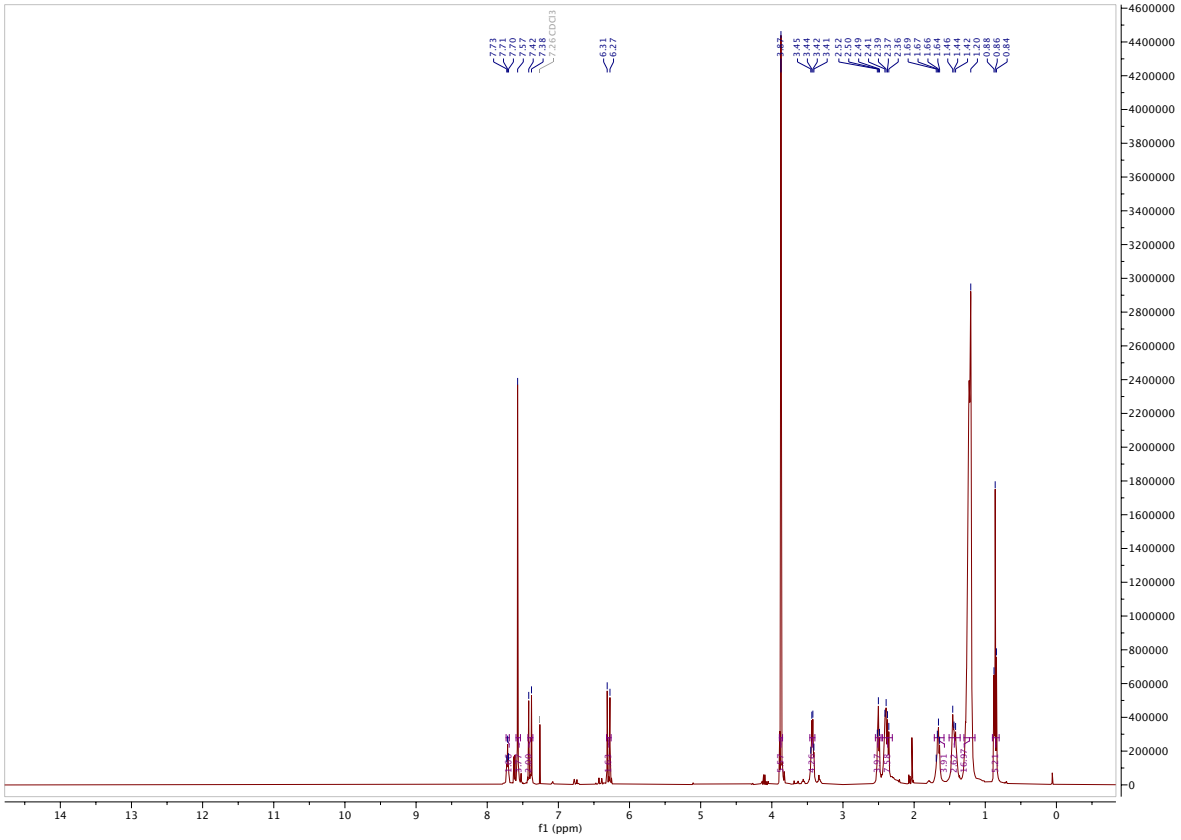


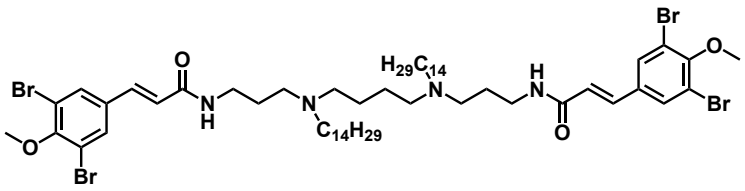

Br
O
N
H
H25C12
N
N
C12H25
H
N
O
Br
O
Br
Br

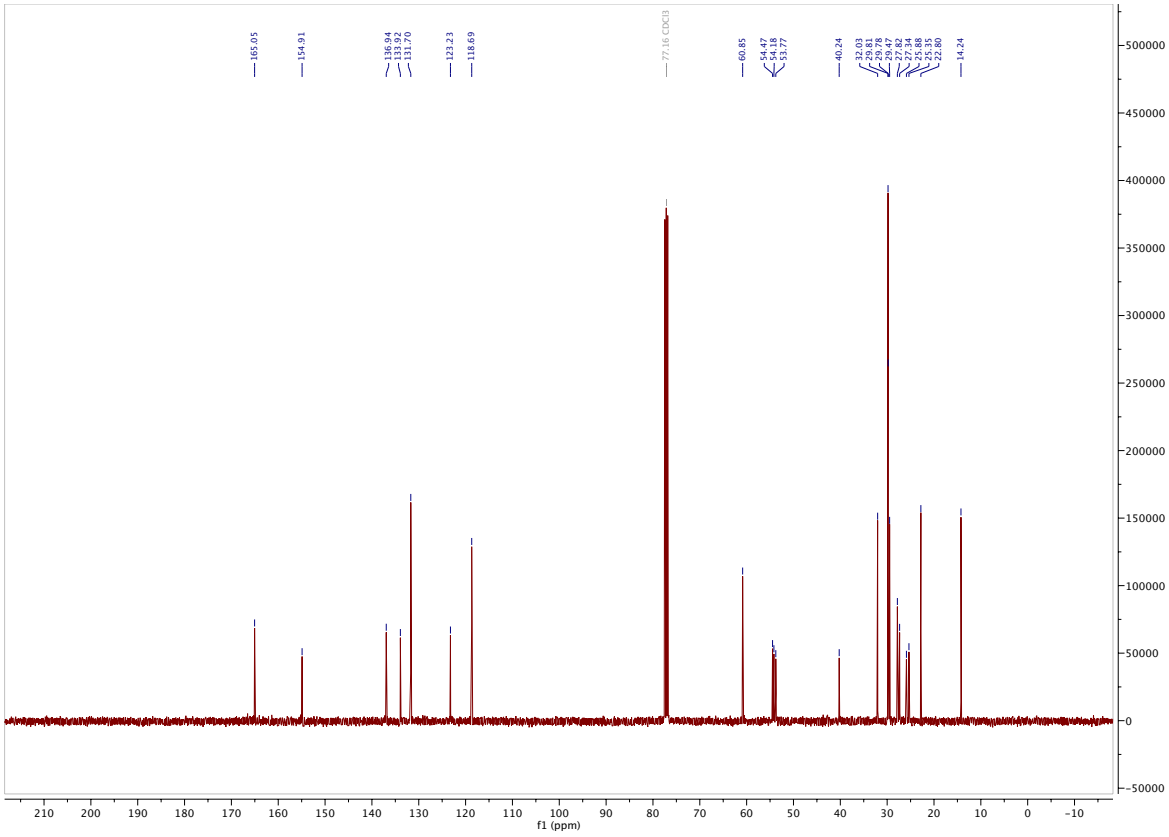

165.04
154.91
136.94
131.92
131.91
131.70
123.23
118.83
118.69
118.64
77.16 CDCl3
60.85
60.51
54.46
54.18
53.77
40.23
32.02
29.81
29.77
29.75
29.46
27.81
27.34
25.88
25.35
22.80
21.16
14.31
14.24
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
-50000
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

(2E,2'E)-N,N'-((butane-1,4-diylbis(tetradecylazanediyl))bis(propane-3,1-diyl))bis(3-(3,5-dibromo-4-methoxyphenyl)acrylamide)






Br
O
N
H
H29C14
N
C14H29
N
H
O
Br
O
Br
Br
O
Br


165.05
154.91
136.94
133.92
131.70
123.23
118.69
77.16 CDCl3
60.85
54.47
54.18
53.77
40.24
32.03
30.81
29.78
29.47
27.82
27.34
25.88
25.35
22.80
14.24
500000
450000
400000
350000
300000
250000
200000
150000
100000
50000
0
-50000
210
200
190
180
170
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
f1 (ppm)

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