

SYNTHESIS AND SCREENING OF APLYSINOPSIN ANALOGS AS POSSIBLE
SEROTONIN RECEPTOR LIGANDS

A DISSERTATION

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BY

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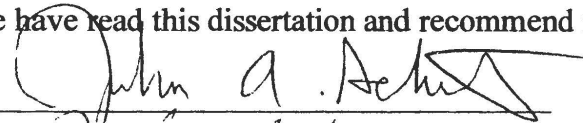
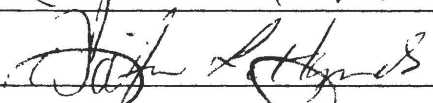
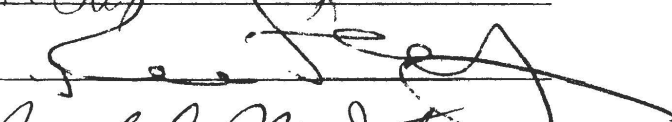
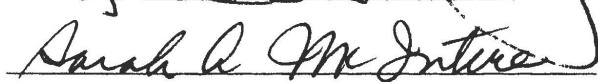

January 8, 2009

To the Dean of the Graduate School:


I am submitting herewith a dissertation written by Diana Catalina Canseco Castillo entitled "Synthesis and Screening of Aplysinopsin Analogs as Possible Serotonin Receptor Ligands." I have examined this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a major in Molecular Biology.


Dr. Lynda Uphouse, Major Professor

We have read this dissertation and recommend its acceptance:






Department Chair

Accepted:


Dean of the Graduate School

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ABSTRACT

DIANA CATALINA CANSECO CASTILLO

SYNTHESIS AND SCREENING OF APLYSINOPSIN ANALOGS AS POSSIBLE SEROTONIN RECEPTOR LIGANDS

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Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine neurotransmitter that modulates a number of functions such as mood, sleep, and sexuality. Because the 5-HT_{2A} and 5-HT_{2C} receptor subtypes are closely related, it has been difficult to develop drugs selective for one or the other subtype. Indole alkaloids (aplysinopsins) isolated from a marine sponge showed a low affinity pharmacophore with selectivity for the 5-HT_{2C} subtype. Our goal for the current work was to exploit this natural product pharmacophore in an effort to create derivative compounds that retain their selectivity but that have improved affinity for the 5-HT_{2C} receptor. Our working hypothesis was that halogenating the indole ring of aplysinopsins would result in derivatives with enhanced affinity and selectivity for the human 5-HT_{2C} receptor subtype.

A series of twenty-two aplysinopsin derivatives were synthesized by carrying out an aldol condensation with creatinine, creatinine derivatives, and indole 3-carboxaldehydes, and tested for their biological activity. All the compounds synthesized were characterized by spectroscopic methods and in some cases by X-ray structural analysis. Compounds were screened for their ability to interact with cloned serotonin receptors, 5-HT_{2A} and 5-HT_{2C}, stably expressed in cell lines. Six compounds of the UNT-TWU series: (*E*)-5-((5-

bromo-1*H*-indol-3-yl)methylene)-2-imino-1,3-dimethylimidazolidin-4-one (**UNT-TWU-8**); (*E*)-5-((6-fluoro-1*H*-indol-3-yl)methylene)-2-imino-1,3-dimethylimidazolidin-4-one (**UNT-TWU-13**); (*E*)-5-((6-chloro-1*H*-indol-3-yl)methylene)-2-imino-1,3-dimethylimidazolidin-4-one (**UNT-TWU-15**); (*E*)-5-((6-bromo-1*H*-indol-3-yl)methylene)-2-imino-1,3-dimethylimidazolidin-4-one (**UNT-TWU-16**); (*Z*)-3-ethyl-5-((fluoro-1*H*-indol-3-yl)methylene)-2-imino-1-methylimidazolidin-4-one (**UNT-TWU-20**); and (*E*)-5-((5,6-dichloro-1*H*-indol-3-yl)methylene)-2-imino-1,3-dimethylimidazolidin-4-one (**UNT-TWU-22**) showed selectivity for cloned human serotonin 5-HT_{2A} or 5-HT_{2C} receptors, while the other compounds tested did not. **UNT-TWU-13** and **UNT-TWU-20** displayed high affinity for the cloned human serotonin 5-HT_{2A} receptor subtype, while **UNT-TWU-15**, **UNT-TWU-16**, the synthetic version of a naturally occurring aplysinopsin (known as NP7), and **UNT-TWU-22** displayed affinity for the cloned serotonin 5-HT_{2C} receptor subtype. Based on these findings, the type and position of halogenation on the indole ring determines selectivity for 5-HT_{2A} and 5-HT_{2C} receptor subtypes. Appropriately halogenated rings can result in high affinity derivatives with selectivities greater than 100-fold.

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LIST OF ABBREVIATIONS

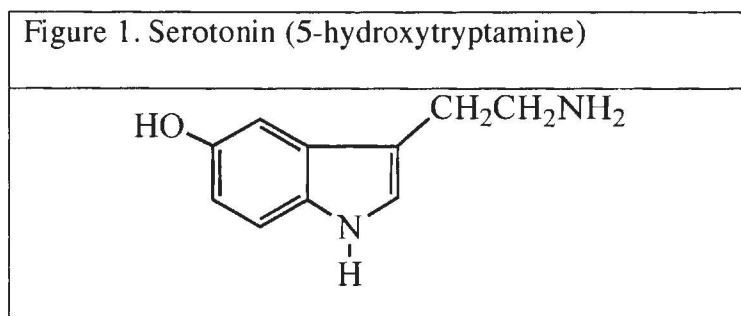
5-HT	5-hydroxytryptamine
ACTH	adrenocorticotrophic hormone
BCA	bicinochonic acid
cAMP	cyclic adenosine monophosphate
d ₆ -DMSO	hexa deuterated dimethyl sulfoxide
DMF	dimethyl formamide
DMSO	dimethyl sulfoxide
DOI	2,5-dimethoxy-4-iodoamphetamine
DOB	2,5-dimethoxy-4-bromoamphetamine
D-PBS	Dulbecco's phosphate buffered saline
EBSS	Earle's balanced salts
GPCR	G-protein-coupled receptor
HEK 293	human embryonic kidney cells
HRMS	high resolution mass spectra
LSD	d-lysergic acid diethylamide
MAO-A	monoamine oxidase A
MCPP	1-(3-chlorophenyl)piperazine
MPPF	4-(2-methoxy)-phenyl-1-[2-(N-2-pyridyl) - <i>p</i> -fluorobenzamido]ethyl-piperazine
mRNA	messenger ribonucleic acid

NaOCH ₃	sodium methoxide
NaOH.....	sodium hydroxide
ORTEP.....	Oak Ridge Thermal Ellipsoid Plot
PLA ₂	phospholipase A ₂
PLC.....	phospholipase C
PLD.....	phospholipase D
RNA.....	ribonucleic acid
SERT	serotonin transporter
SNP	single nucleotide polymorphism
THF.....	tetrahydrofuran
TLC.....	thin layer chromatograph

CHAPTER I
INTRODUCTION

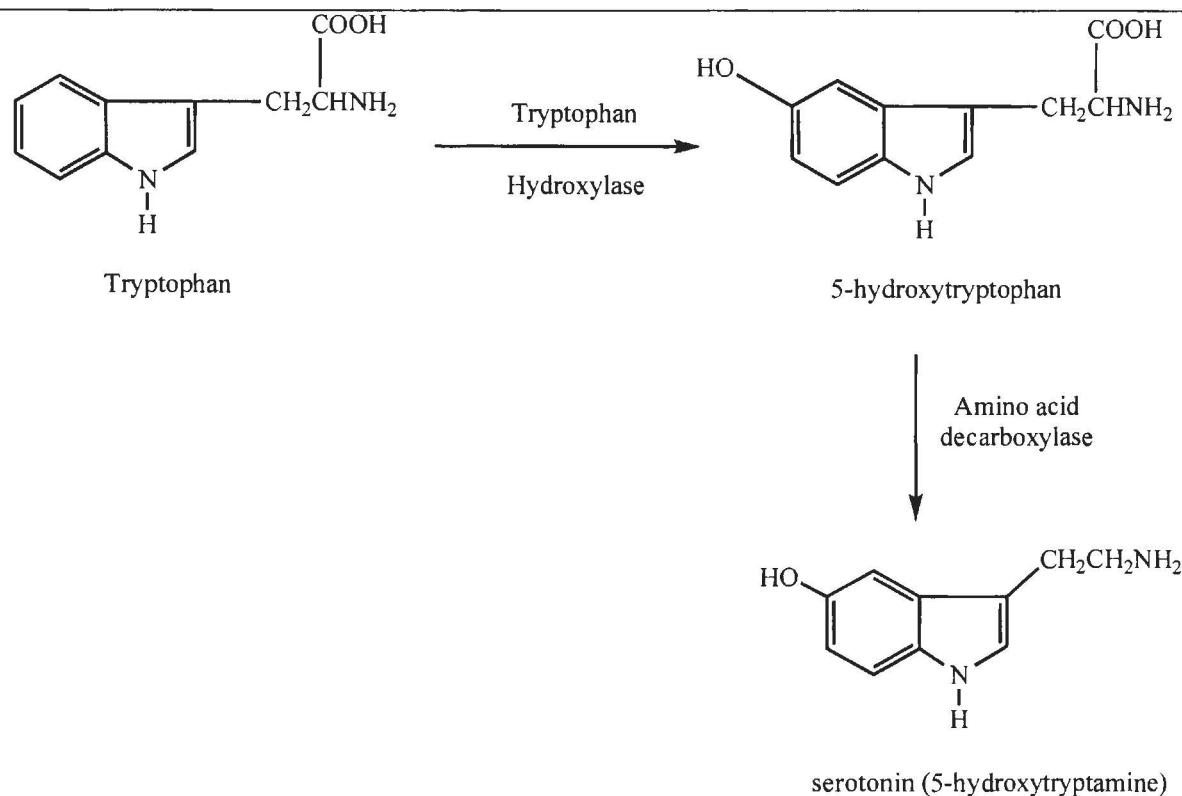
Serotonin

Serotonin is an indolealkylamine whose systematic name is 3-(2-aminoethyl)-1*H*-indol-5-ol and is commonly known as 5-hydroxytryptamine (5-HT). The chemical structure of 5-HT (Figure 1) reveals its close relationship to the amino acid, tryptophan, which serves as the precursor of the neurotransmitter¹.



Serotonin is synthesized in cells from tryptophan in a two-step process. In the first step, which is rate limiting, tryptophan is hydroxylated in the 5-position. In the second step, 5-hydroxytryptophan is decarboxylated to serotonin (Scheme I)¹.

Scheme I



Serotonin, released from nerve terminals, is taken up by a serotonin transporter (SERT) and returned to the vesicular pool via the vesicular monoamine transporter 2 (VMAT2) or subsequently degraded by monoamine oxidase A (MAO-A). 5-HT is involved in numerous functions in the mammalian central nervous system. These functions are mediated by a large number of receptors¹.

Serotonin Receptors

The study of 5-HT receptors has resulted in the identification of seven families (5-HT1-5-HT7) and several subtypes of 5-HT receptors^{2,3}. The serotonin receptor superfamily is composed of 14 members classified on the basis of gene structure, amino acid sequence homology, and intracellular signaling cascades⁴. Except for the 5-HT3 receptor, which is a ligand-gated channel receptor, activation of these receptors leads to production of second messengers that regulate cellular functions via phosphorylation / dephosphorylation of intracellular proteins. These families regulate the two major intracellular second messenger pathways, adenylate cyclase and phospholipase C^{5,6}.

The six G protein-coupled (GPCR) 5-HT receptors, 5-HT1, 5-HT2, 5-HT4, 5-HT5, 5-HT6 and 5-HT7, are divided into 13 families. The 5-HT1 receptor family contains the 5-HT1A, 5-HT1B, 5-HT1D, 5-HT1E and 5-HT1F receptors. The 5-HT2 receptor family contains the 5-HT2A, 5-HT2B and 5-HT2C receptors. The 5-HT5 receptor family contains 5-HT5A and 5-HT5B receptors. For the most part, the 5-HT4, 5-HT6, and 5-HT7 families do not contain subtypes of receptors and sequence diversity in these families is provided mainly by alternative mRNA splicing.

RNA editing provides additional sequence diversity in some families of 5-HT receptors and numerous single nucleotide polymorphisms (SNPs) and splice variants are known to exist in these receptors^{7,8}. The focus of this study was the 5-HT2A and 5-HT2C receptors.

The 5-HT_{2A} and 5-HT_{2C} Receptors

5-HT₂ receptors mediate the actions of drugs used in the treatment of disorders such as schizophrenia, eating disorders, depression, migraine, hypertension, anxiety, and gastrointestinal dysfunctions¹. These two receptors are closely related and belong to the rhodopsin family of the G-protein coupled receptors (GPCRs)^{1,2}. GPCRs have been initially associated with activation of phospholipase C (PLC) via Gq-protein. The primary signaling linkage of the 5-HT_{2A} receptor is to the activation of PLC- β in nearly all tissues and cells in which it is expressed⁹. Hydrolysis of phosphoinositides and elevation of intracellular Ca²⁺ occur in response to activation of PLC⁷. This receptor increases intracellular Ca²⁺ levels through several mechanisms, including the liberation of intracellular Ca²⁺ stores or by activation of Ca²⁺ channels. The 5-HT_{2A} receptor can also modulate other signals, such as the activation of phospholipases including phospholipase D (PLD) and phospholipase A₂ (PLA₂). The 5-HT_{2A} receptor can also regulate cAMP formation in certain cells⁸. For example, in renal cells, the 5-HT_{2A} receptor inhibits forskolin-stimulated cAMP formation through a pertussis toxin-sensitive mechanism⁹.

5-HT_{2C} receptors have been reported to modulate a variety of secondary signals. Activation of 5-HT_{2C} receptors leads to the inhibition of K⁺ channels in the choroid plexus¹⁰ and, when expressed in cell lines, the 5-HT_{2C} receptor can activate PLA₂-mediated arachidonic acid release¹⁰. The 5-HT_{2C} receptor can also regulate cAMP production under certain circumstances. When the receptor was expressed at a high density in stably transformed AV12 cells, it was shown to inhibit forskolin-stimulated

cAMP production through $G_{i/o}$ proteins. When expressed at a low density, the 5-HT_{2C} receptor was shown to potentiate forskolin-stimulated cAMP production by about two fold, probably through the release of G protein $\beta\gamma$ -subunits¹¹. The 5-HT_{2C} receptor can also modulate a number of distinct transport processes. Recombinant 5-HT_{2C} receptors expressed in 3T3 cells can stimulate amyloid precursor protein processing through a pathway that involves PKC and PLA₂¹². The 5-HT_{2C} receptor activates an electrogenic Na⁺/Ca²⁺ exchanger in histaminergic neurons in the tuberomammillary nucleus of the hypothalamus, resulting in depolarization and increased firing rates of the neurons¹³. The 5-HT_{2C} receptor has been implicated in the secretion of corticosterone and adrenocorticotrophic hormone (ACTH)¹⁴. The 5-HT_{2C} receptor has also been reported to inhibit the neuronal release of norepinephrine and dopamine, but not 5-HT¹³.

The 5-HT_{2C} receptor exhibits a novel mechanism for generating multiple functional receptor variants through a process called mRNA editing. Burns *et al*¹⁵. observed that five adenosines (sites A-E) within the coding sequence of the putative second intracellular loop (i2) of the 5-HT_{2C} receptor were converted to guanosines at the RNA level. The mRNA editing probably occurs by deamidation of the adenosines to inosines, which are then read at the RNA level as guanosines¹⁶. This phenomenon appears to be unique for the 5-HT_{2C} receptor among GPCRs. Neither the 5-HT_{2A} nor the 5-HT_{2B} receptors or other GPCRs have been shown to undergo mRNA editing¹⁷. The functional significance of the various edited isoforms of the 5-HT_{2C} receptor has been underscored by their differential abundance of expression in total brain and

hypothalamic mRNA¹⁸ and their distinct functional properties. Moreover, the variant edited receptors exhibit differential abilities to bind various ligands, to mobilize intracellular Ca^{2+} and to stimulate hydrolysis of inositol phosphates^{17,18}. Burns and colleagues showed that mRNA editing of the 5-HT_{2C} receptor leads to a 10 to 15 fold reduction in the efficacy of the interaction between receptors and their G proteins¹⁵. Those observations led to the speculation that mRNA editing plays an important role in determining the specificity of signaling by the 5-HT_{2C} receptor.

The 5-HT₂ receptor subtypes are targets for a wide array of drugs including antipsychotics, antidepressants, and antihistamines, which have 5-HT₂ receptor antagonistic action, and to agonistic hallucinogens such as d-lysergic acid diethylamide (LSD), methyl-indole-ethylamine derivatives, methoxy-substituted phenylaminopropanes (eg 2,5-dimethoxy-4-iodoamphetamine (DOI) and 2,5-dimethoxy-4-bromoamphetamine (DOB)), and compounds inducing panic attacks (such as 1-(3-chlorophenyl)piperazine, mCPP)⁶.

In summary, the 5-HT₂ receptors have very similar pharmacological profiles and only a few selective ligands are available. However, differences in signal transduction characteristics of these receptors have been reported⁴.

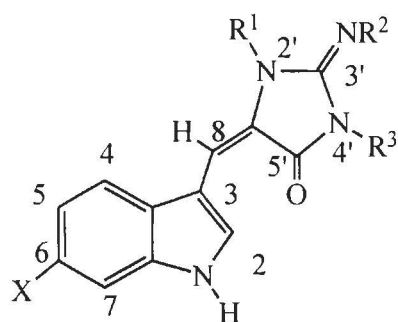
Approach to Differentiation Between 5-HT_{2A} and 5-HT_{2C} Receptors

To understand how 5-HT can modulate complex behaviors, one must be able to dissect the contributions of individual receptor subtypes to these behaviors. The classical approach has been to find selective drugs that either block or activate certain receptor

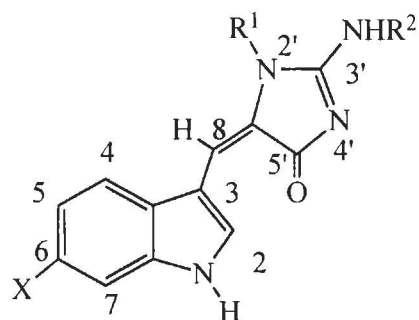
subtypes, and then to study the effects of these compounds on physiology and behavior¹⁹. Several functional parameters (including hypolocomotion, hyperthermia, anxiety and sexual activity) have been proposed for the characterization of actions at 5-HT_{2C} sites⁵. Compounds relatively selective for 5-HT_{2A} receptors lead to head twitches and wet dog shakes²⁰, but without a selective agonist/antagonist, the true validity of these parameters is difficult to determine. The purpose of this research is to develop synthetic methods for the preparation of compounds that will selectively bind to the human serotonin 5-HT_{2A} or 5-HT_{2C} receptor subtypes. These compounds could also bind to other receptors, but this study will be limited to a determination of their selectivity between 5-HT_{2A} and 5-HT_{2C} subtypes. If a compound is selective for the 5-HT_{2A} receptor, it has the potential of eventually being used for the treatment of obesity (agonist) or depression (antagonist). If a compound is selective for the 5-HT_{2C} receptor, it has the potential of eventually being used for the treatment of male sexual dysfunction (agonist).

In 2002, Hu *et al.* determined 5-HT_{2A} and 5-HT_{2C} receptor subtype binding affinities and selectivity ratios for a number of indoleamine alkaloid natural products isolated from *Verongida* marine sponges (Figure 2)²¹. These compounds, called aplysinopsins, are structurally related to serotonin in that they contain an indole ring and a basic nitrogen atom (3' position in imidazolidine ring) positioned approximately 4 angstroms from the number 3 position of the indole ring (Figure 3) (Dr. James E. Johnson personal communication).

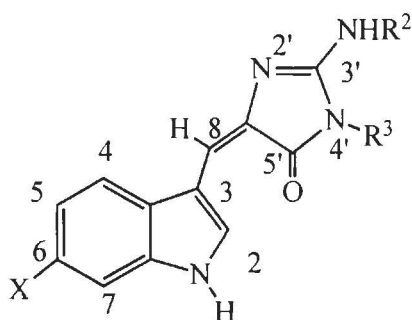
Figure 2. Indoleamine alkaloid natural products (1-8) isolated from *Verongida* marine sponges (called Aplysinopsins)



- 1: X = R² = H; R¹ = R³ = CH₃
 2: X = Br; R¹ = R³ = CH₃; R² = H
 3: X = H; R¹ = R² = R³ = CH₃
 4: X = H; R¹ = R³ = CH₃; R² = CH₂CH₃

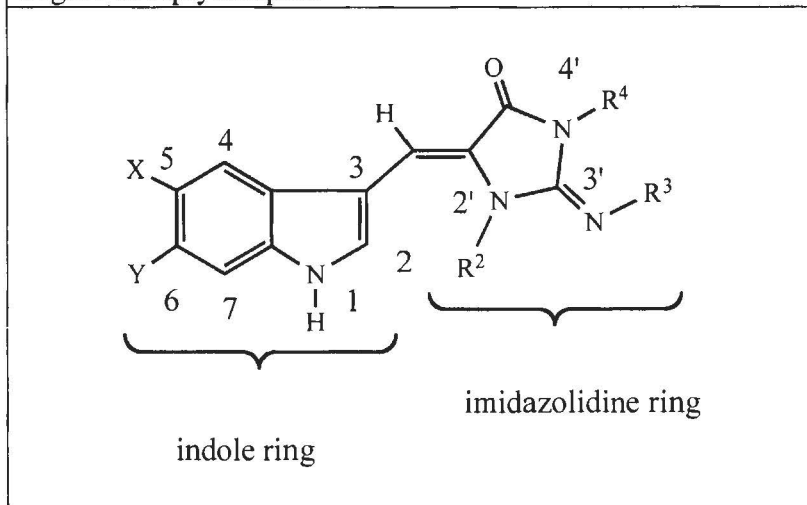


- 5: X = H; R¹ = R² = CH₃
 6: X = Br; R¹ = CH₃; R² = H



- 7: X = R² = H; R³ = CH₃
 8: X = Br; R² = H; R³ = CH₃

Figure 3. Aplysinopsin

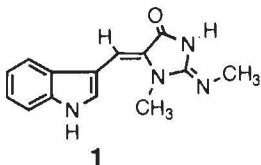
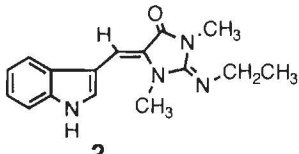
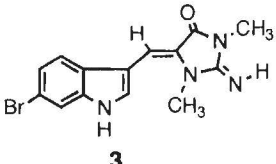
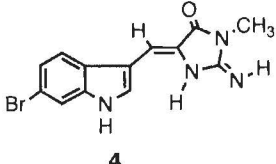


The aplysinopsin **4** (Table 1) has low binding affinities but shows a high selectivity for 5-HT_{2C} receptors. This aplysinopsin has a bromine atom in the 6-position of the indole ring and a methyl group on the 4' position of the imidazolidine ring (Table 1)²¹. A compound with a bromine atom in both the 5 and 6 positions has high affinity but was not selective. An ethyl group on the imine nitrogen at the 3' carbon atom improved affinity but reduced selectivity.

The current research was designed to synthesize product pharmacophores related to the natural compound that might have therapeutic potential. The specific aim was to synthesize compounds with both high affinity and selectivity for the serotonin 5-HT_{2C} receptor subtype. A variety of non-naturally occurring analogs of the most 5-HT_{2C} receptor selective aplysinopsin (compound **4**, see Table 1) were synthesized and then

screened for specific high affinity binding to cloned human cells containing 5-HT_{2A} and 5-HT_{2C} receptor subtypes. It was hypothesized that the affinity and selectivity for the human 5-HT_{2C} receptor subtype would be enhanced in aplysinopsin derivatives with halogen atoms in the 6 position of the indole ring and a methyl group on the 4' position of the imidazolidine ring system.

Table 1. Human serotonin 5-HT_{2A} and 5-HT_{2C} receptor subtype affinities and selectivity ratios for indoleamine alkaloid natural products isolated from Verongida marine sponges as modified from Hu et al.²¹.

Compound	K _i (nM)		K _i , Selectivity Ratio (5-HT _{2A} /5-HT _{2C})	Comments
	5-HT _{2A}	5-HT _{2C}		
 1	NB	NB	—	
 2	1,700	3,500	0.5	3' ethylation improves affinity, but reduces selectivity
 3	2,000	330	6.0	2' alkylation increases affinity, but reduces selectivity
 4	>100,000	2,300	>40	most selective

NB = Not bound

CHAPTER II

METHODS

Synthetic Procedures

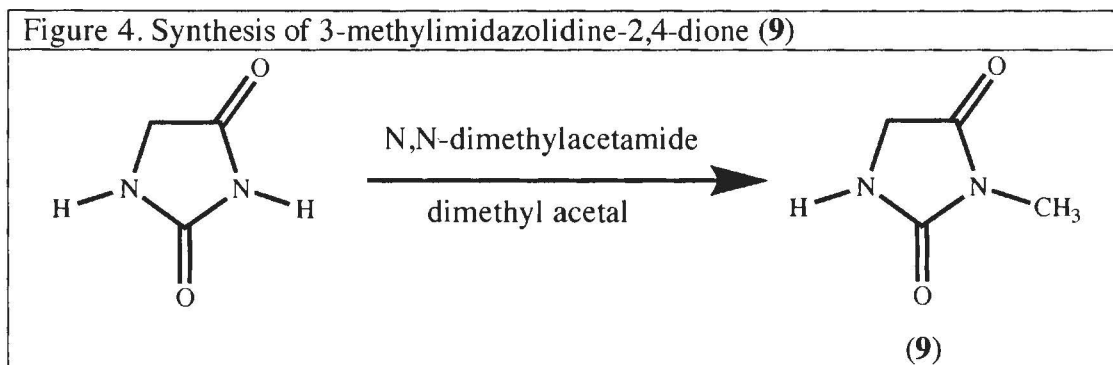
General Methods

All chemicals used in this research were reagent grade unless otherwise specified. Melting points were determined in an OptiMelt Automated Melting Point System by Stanford Research Systems (Sunnyvale, CA) or a Mel-Temp (from Sigma-Aldrich) and are uncorrected. Column chromatography was performed using Silica 200-300 mesh from Natland International Corporation (Research Triangle Park, NC). Infrared spectra were determined on nujol mulls samples between sodium chloride plates using a Nicolet Magna 560 FTIR (Madison, WI) over the frequency range of 4000-600 cm^{-1} . ^1H NMR spectra were obtained in hexa deuterated dimethyl sulfoxide (d_6 -DMSO) on a Varian Mercury 300 MHz spectrometer (Palo Alto, CA). Identification of compounds using high resolution mass spectra (HRMS) was contracted to the University of Minnesota using either a Finnigan MAT 95 or Bruker BioTOF mass spectrometer. Purification of solid samples was done by recrystallization unless otherwise specified. Briefly, the process of recrystallization involves dissolving the solid in an appropriate solvent at elevated temperature and allowing the crystals to re-form on cooling, so that any impurities remain in solution.

A purified compound from the natural product aplysinopsin was utilized as the model for the synthetic scheme for this research project (Figure 3). This purified natural product was selected based on previous reports by Hu *et al*²¹, that indicated that this aplysinopsin had selectivity between 5-HT_{2A} and 5-HT_{2C} receptors.

Synthesis of 3-methylimidazolidine-2,4-dione (9)

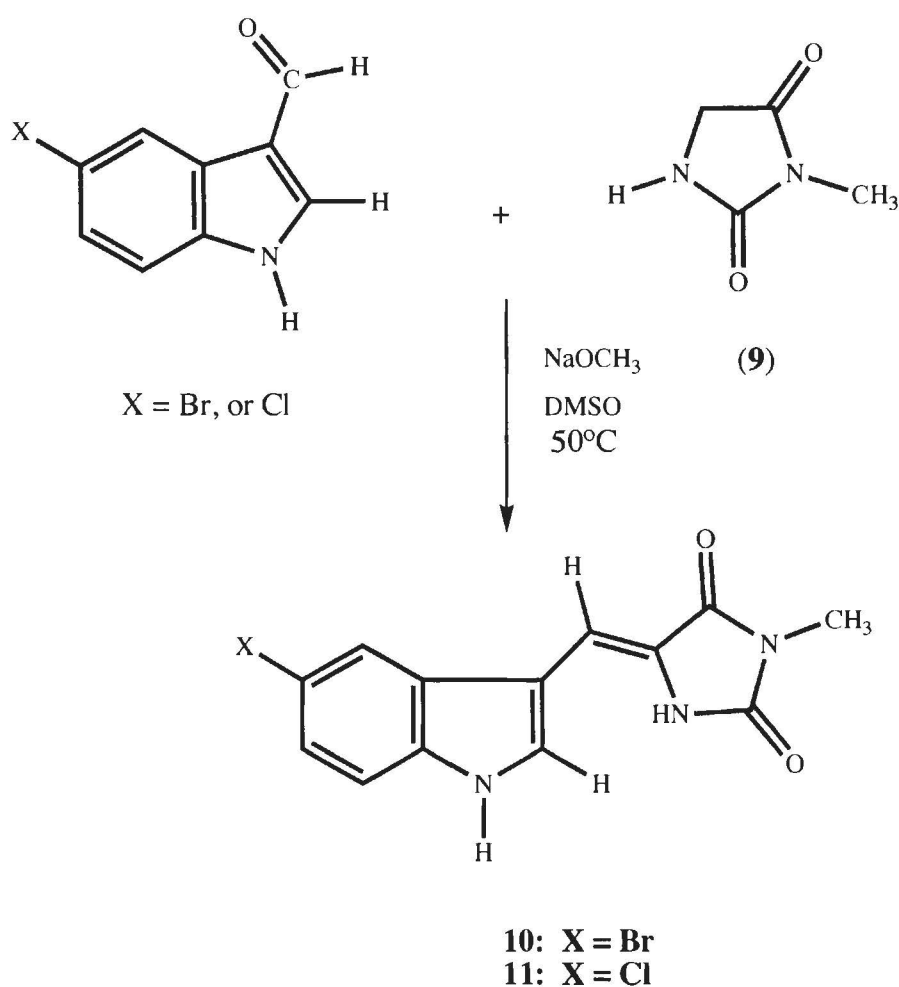
A procedure by Arenal was used for the synthesis of (9) (See Figure 4)²². Hydantoin (1.0 g) and N,N-dimethylacetamide dimethyl acetal (4.0 g) were refluxed for 3 h in toluene (50 mL). After cooling, the solid that formed was filtered by suction filtration to give 1-methylimidazol-2,5 (1, 3H)-dione. The product was recovered as a yellow solid, with a 36% yield (0.35 g) and an m.p. of 181-182 °C²².



Compound (9) was used without further purification for the aldol condensation from the 5-bromoindole-3-carboxaldehyde and the 5-chloroindole-3-carboxaldehyde (Figure 5). The 5-bromoindole-3-carboxaldehyde and the 5-chloroindole-3-

carboxaldehyde were reacted with NaOCH₃ and DMSO at 50 °C to produce compounds **10** and **11**.

Figure 5. Synthesis of 5-bromo or 5-chloro (1H-indol-3yl)methylene)-3-methylimidazolidine-2,4-dione (**10** and **11**)



At this point a new procedure was found that allowed us to synthesize the aplysinopsin analogs required for this research. However, the indole aldehydes required

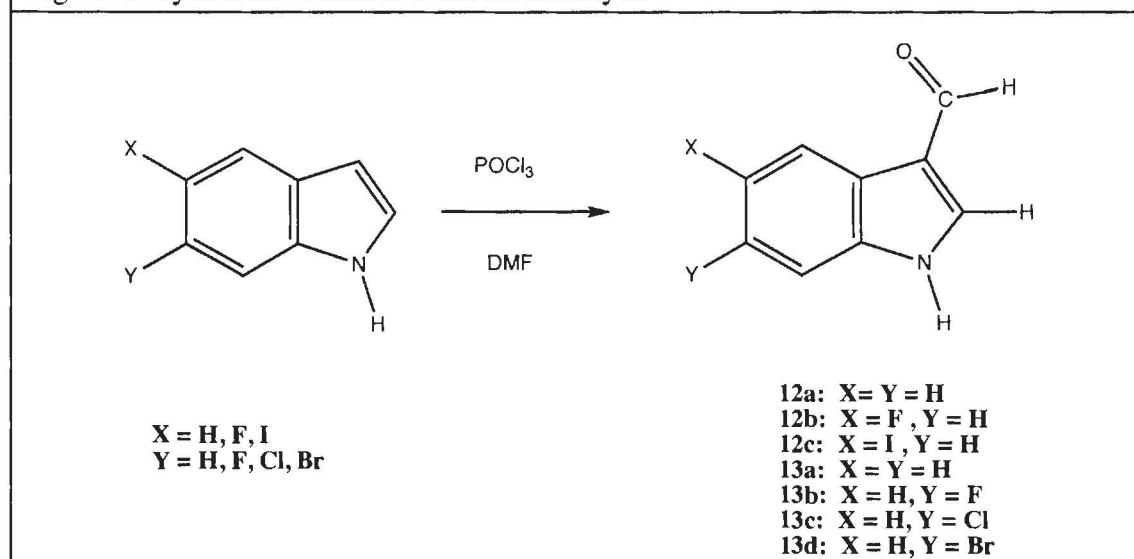
for this synthesis were not commercially available, which necessitated a procedure to make these aldehydes. It was decided to start with the synthesis of the indole aldehydes.

General Procedure for the Synthesis of indole-3-carboxaldehydes

(12a, 12b, 12c, 13a, 13b, 13c, 13d) (Figure 6)

The procedure used for this synthesis was reported by Jiang *et al*²³. Phosphorus (V) oxychloride (4.2 g) was added dropwise to dimethyl formamide (DMF) (6 mL) and cooled in an ice bath. The mixture was kept at 0 °C for approximately 30 min. A solution of the indole (3.0 g) in DMF (22 mL) was added dropwise, keeping the reaction mixture below 10 °C. After 3 h at 20 °C, the solution was poured into an ice-water mixture (180 g), neutralized with 1N (NaOH), and left overnight at room temperature. The crude product was collected by filtration and recrystallized from ethanol/water to give the indole-3-carboxaldehyde.

Figure 6. Synthesis of indole-3-carboxaldehyde



Synthesis of 5-fluoroindole-3-carboxaldehyde (12b)

Phosphorus(V) oxychloride (4.3 g), DMF (6 mL) and 5-fluoroindole (3.0 g) gave 5-fluoroindole-3-carboxaldehyde (2.2 g, 72%); m.p. 170 – 171 °C (reported m.p. 170 - 171 °C)²⁴.

Characterization of this compound via proton NMR (¹H-NMR) was as follows:

¹H-NMR: δ 12.29 (s, 1H, NH), 9.93 (s, 1H, CH=O), 8.37, (s, 1H, C(2)H), 7.77 (dd, J = 3 Hz and J = 10 Hz, 1H, C(4)H), 7.54 (dd, J = 5 Hz, and J = 9 Hz, 1H, C(7)H), 7.13 (tt, J = 9 Hz and J = 3 Hz, 1H, C(6)H).

Synthesis of 5-iodoindole-3-carboxaldehyde (13c)

Phosphorus (V) oxychloride (3.8 g), DMF (6 mL) and a solution of the 5-iodoindole (5.0 g) in DMF (22 mL) gave 5-iodoindole-3-carboxaldehyde as a light yellow solid (3.0 g, 78% yield).

Synthesis of 5,6-dichloroindole-3-carboxaldehyde

The synthesis of 5,6-dichloroindole-3-carboxaldehyde involved 4 steps. The first step was the nitration of the 3,4-dichlorotoluene; step 2 was the synthesis of (E)-1-(4,5-dichloro-2-nitrostyryl)-pyrrolidine. Step three was the synthesis of the 5,6-dichloroindole; step four was the synthesis of the 5, 6-dichloroindole-3-carboxaldehyde.

Step 1: Nitration of 3,4-dichlorotoluene. A solution of sulfuric acid (11 mL) and nitric acid (11 mL) were mixed together in a three-neck round bottomed flask and cooled to room temperature. Through a dropping funnel, 3,4-dichlorotoluene (20 g) was slowly added to the solution mixture. The temperature was kept below 50 °C using a cold-water bath. After addition was completed and the exothermic reaction had subsided, the reaction was stirred and heated at 55 °C for 25 min. The reaction was cooled to room temperature and then poured into 20 mL of cold water. The solid that formed was filtered and it was recrystallized three times using methanol:water. A yellow solid (6.4 g) was recovered and used for step two.

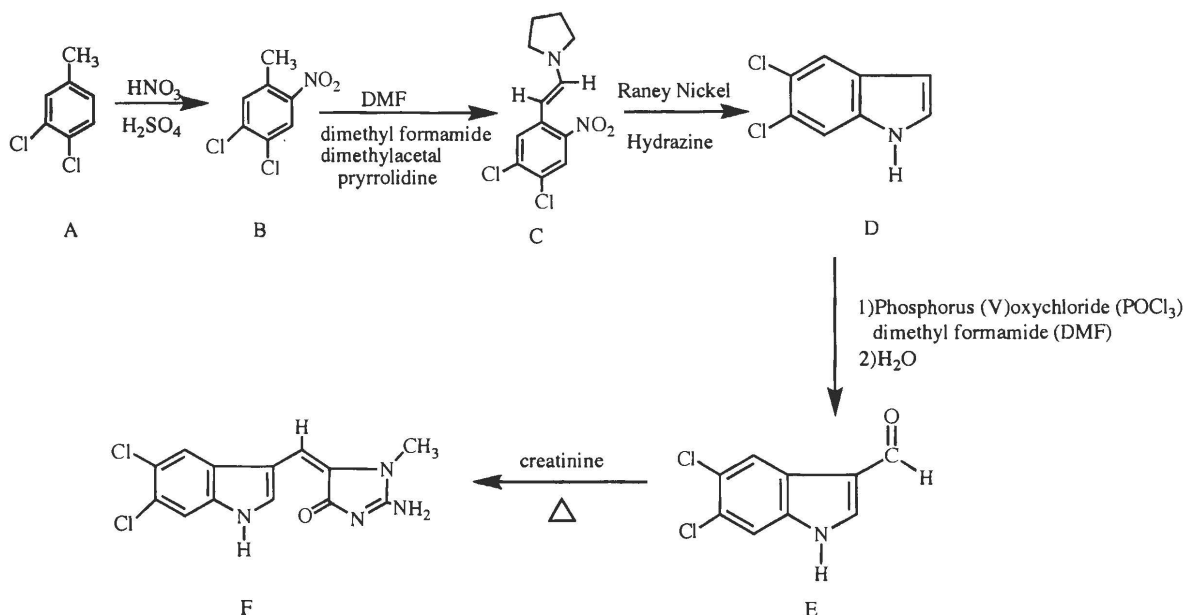
Step 2: Synthesis of (E)-1-(4,5-dichloro-2-nitrostyryl)-pyrrolidine. To a solution of 1,2-dichloro-4-methyl-5-nitrobenzene (6.4 g) in 17 mL of DMF, N,N-dimethylformamide dimethyl acetal (4.3 g) and pyrrolidine (2.5 g) were added to the reaction mixture. The solution was heated, under nitrogen gas, to 110 °C for 3 h. The reaction mixture was cooled to room temperature and the volatile components were evaporated via rotary evaporation. A red solid was collected that was later dissolved in 9 mL of methylene chloride and 70 mL of anhydrous methanol. The solution mixture was once again rotary evaporated and a red solid was collected. The red solid (2.5 g) was used without further purification for step three.

Step 3: Synthesis of 5,6-dichloroindole. One mL of raney nickel was added to a solution of (E)-1-(4,5-dichloro-2-nitrostyryl)-pyrrolidine (2.5 g) in 18 mL of tetrahydrofuran (THF) and 18 mL of methanol, under nitrogen gas. This solution mixture was heated to 30 °C. Hydrazine hydrate (0.82 g) was added to the solution and a vigorous gas evolution was observed. At this point, the temperature was increased to 45 °C. After 30 min, an additional 0.82 g hydrazine hydrate was added followed by another 0.82 g after 1 h. The reaction was heated for a total of 2 h following the last addition of hydrazine hydrate. The reaction mixture was cooled to room temperature, and the catalyst was removed by filtration through celite and washed several times with anhydrous methanol. The filtrate was evaporated and the residue was dried. A dark red/black solid was collected. This solid was purified using column chromatography with 50:50 chloroform:hexane mixture as the eluent. The 5,6-dichloroindole was collected as a white powder.

Step 4: Synthesis of the 5,6-dichloroindole-3-carboxaldehyde. The indole carboxaldehyde was prepared using the procedure described earlier (Figure 6) under the heading of general procedure for the synthesis of indole-3-carboxaldehydes.

The synthesis scheme for the synthesis of 5,6-dichloroindole-3-carboxaldehyde is shown in Figure 7.

Figure 7. Synthesis of 5,6-dichloroindole-3-carboxaldehyde



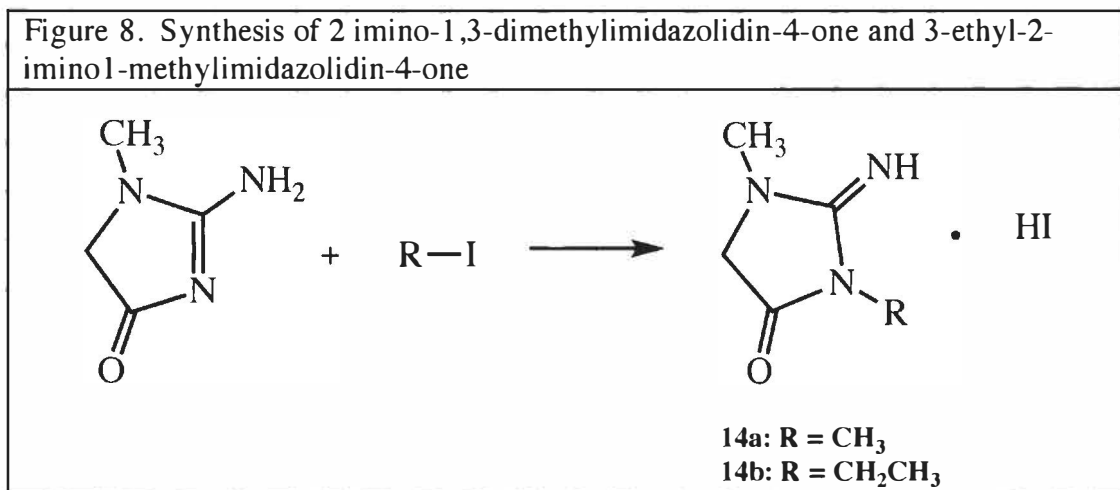
Synthesis of 2-amino-1,3-dimethylimidazolidin-4-one (14a)

The procedure used for the synthesis of 2-amino-1,3-dimethylimidazolidin-4-one was reported by Kenyon and Rowley²⁵. Creatinine (10 g) and iodomethane (15.0 g) were dissolved in 85 mL of 95% ethanol and refluxed for 3.5 h. The reaction mixture was cooled to room temperature and the solvent was removed by rotary evaporation. The crude product was dissolved in methanol and passed through a basic ion-exchange resin column (Bio-Rad AG 3-X4 washed with 200 mL of anhydrous methanol prior to use). After 300 mL of eluent was collected, the methanol was removed by rotary evaporation.

The product was extracted with benzene (3 x 30 mL) and the benzene was removed by rotary evaporation to give a pale yellow solid.

Synthesis of 2-amino-3-ethyl-1-methylimidazolidin-4-one (14b)

The procedure used for the synthesis of 2-amino-3-ethyl-1-methylimidazolidin was a modification of that reported by Kenyon and Rowley²⁵. Creatinine (10 g) and iodoethane (22.1 g) were dissolved in 100 mL of 95% ethanol and refluxed for 7 days. The solvent was removed by rotary evaporation. The crude product was dissolved in anhydrous methanol and passed through a basic ion exchange column as previously described. After 300 mL of eluent was collected, the methanol was removed by rotary evaporation and the product was extracted with benzene (2 x 30 mL). The benzene was removed by rotary evaporation. The crude product was recrystallized three times with acetonitrile to give a light yellow solid (Figure 8).



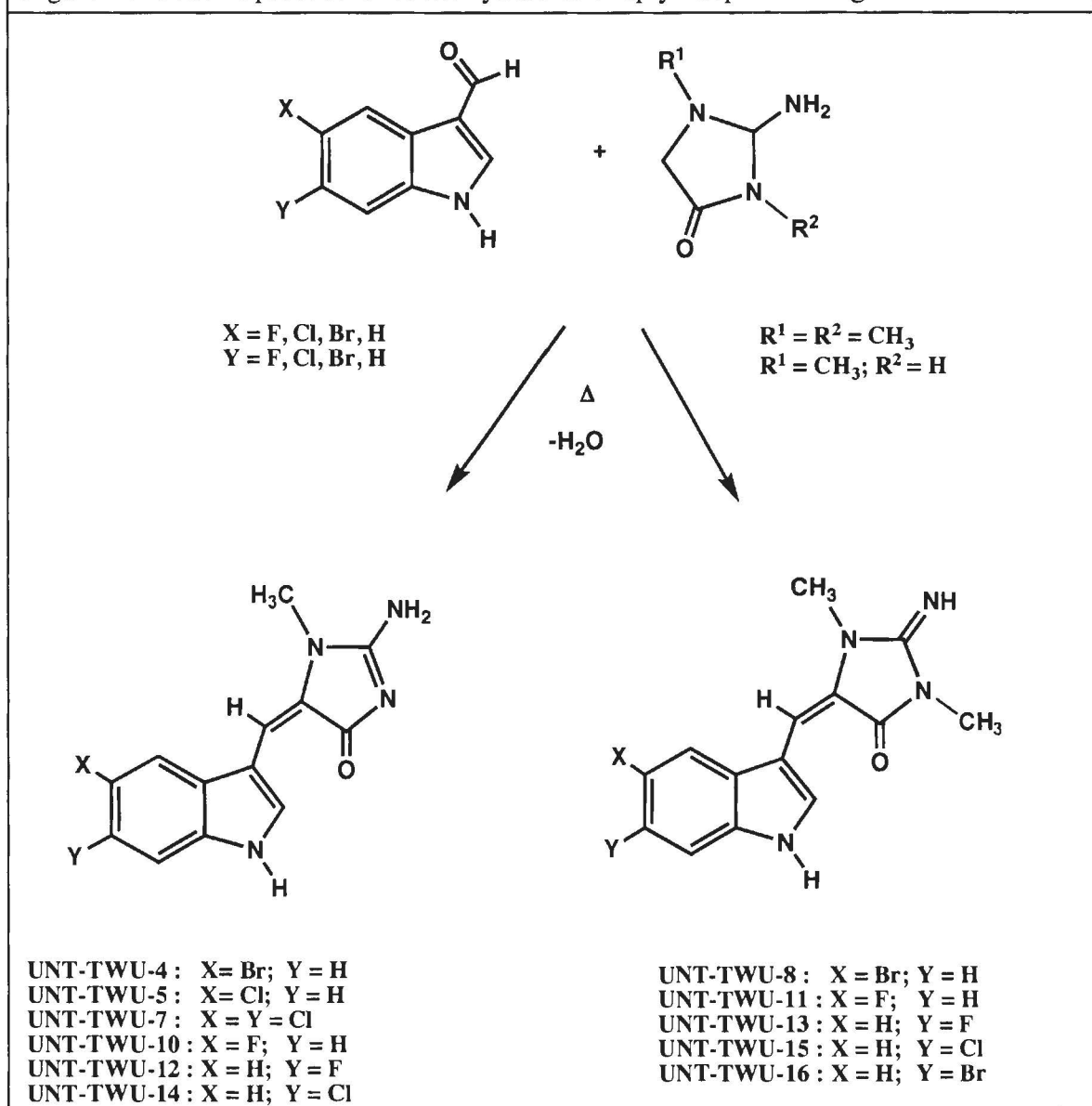
General Procedures for the Reaction of indole aldehydes with creatinine, 2-amino-1,3-dimethylimidazolidin-4-one (14a) or 2-amino-3-ethyl-1-methylimidazolidin-4-one (14b)

The procedure for the reaction of 2-amino-1,3-dimethylimidazolidin-4-one (**14a**) or 2-amino-3-ethyl-1-methylimidazolidin-4-one (**14b**) was originally reported by Djura *et al*²⁶. Indole-3-carboxaldehyde and creatinine were placed in a 100 mL three-neck round-bottomed flask fitted with a nitrogen gas inlet and a probe from a JKEM 210 for monitoring the temperature. The flask was then heated cautiously with a Bunsen burner and the temperature was kept at approximately 153 °C for 10 min. Considerable frothing of the mixture took place during the course of the reaction. The reaction mixture was allowed to cool to room temperature and then extracted with methanol (2 x 50 mL). The residual solid was recrystallized (Figure 9).

Synthesis of 2-amino-5-((5-bromo-1H-indol-3-yl)methylene)-1-methyl-1,5-dihydroimidazol-4-one (UNT-TWU-4)

5-Bromoindole-3-carboxaldehyde (5.0 g) and creatinine (2.5 g) gave UNT - TWU-4. The compound was recrystallized from DMF-water to give light orange needles (2.3 g, 32% yield), m.p 309-310 °C (decomposition begins at 280 °C). High resolution mass spectra (HRMS): $M^{++} + H$, 319.0201. Requires $M^{++} + H$, 319.0189.

Figure 9. General procedure for the synthesis of aplysinopsin analogs



Selected infrared absorptions (cm^{-1}): 3318, 3163, 1696, 1661, 1624 cm^{-1} .

Characterization of this compound via proton NMR (^1H -NMR) was as follows:

¹H-NMR: δ 11.63 (s, 1H, NH), 9.13 [d, *J* = 2.7 Hz, 1H, C(2)H], 8.19 [d, *J* = 1.8 Hz, 1H, C(4)H], 7.96 (s, DMF), 7.73 (s, 2H, NH₂), 7.40 [d, *J* = 9.0 Hz, 1H, C(7)H], 7.26 [dd, *J* = 8.4 Hz and *J* = 2.1 Hz, 1H, C(6)H], 6.55 [s, 1H, C(8)H], 3.30 (s, 3H, CH₃).

¹³C-NMR: δ 175.49, 165.13, 162.34 (DMF), 134.33, 131.27, 129.89, 129.67, 124.10, 120.73, 113.77, 112.40, 108.98, 104.25, 35.80, 30.79 (DMF), 27.95 (DMF).

Synthesis of (E)-2-amino-5-((5-chloro-1H-indol-3-yl)methylene)-1-methyl-1H-imidazol-4(5H)-one (UNT-TWU-5)

5-Chloroindole-3-carboxaldehyde (5.0 g) and creatinine (3.4 g) gave UNT - TWU-5. Recrystallization from DMF-water gave an orange solid (2.6 g, 54% yield), m.p. 208 °C (dec) HRMS: M⁺ + H, 275.0691. Requires M⁺ + H, 275.0694.

Characterization of this compound via proton NMR (¹H-NMR) was as follows:

¹H-NMR: δ 11.63 (s, 1H, NH), 9.13 (s, 1H, C(2)H), 7.96 (s, 1H, C(4)H), 8.05 (s, DMF), 7.73 (s, 2H, NH₂), 7.26 [dd, *J* = 8.4 Hz, 1H, C(6)H], 6.55 (s, 1H, C(8)H), 3.30 (s, 3H, CH₃), 2.86 (s, DMF), 2.70 (s, DMF)

Synthesis of (E)-2-amino-5-((5,6-dichloro-1H-indol-3-yl)methylene)-1-methyl-1H-imidazol-4(5H)-one (UNT-TWU-7)

5,6-Dichloroindole-3-carboxaldehyde (1.0 g) and creatinine (0.55 g) gave **UNT-TWU-7**, a red solid (0.61 g, 61% yield), m.p. 190 °C (dec). HRMS: $M^{+} + H$, 309.0303. Requires $M^{+} + H$, 309.0304.

Selected infrared absorptions (cm^{-1}): 3457, 3173, 2719, 1675, 1639, 1608, 1531 cm^{-1}

Characterization of this compound via proton NMR (^1H -NMR) was as follows:

^1H -NMR: δ 11.68 (s, 1H, NH), 9.15, [d, $J = 2.4$ Hz, 1H, C(9)H], 8.28 (s, 1H, C(4)H), 7.68 (s, 1H, C(7)H), 6.54 (s, 1H, C(8)H), 3.29 (s, 2H, CH_3)

Synthesis of (E)-5-((5-bromo-1H-indol-3-yl)methylene)-2-imino-1,3-dimethylimidazolidin-4-one (UNT-TWU-8)

5-Bromoindole-3-carboxaldehyde (3.7 g) and 2-amino-1,3-dimethylimidazolidin-4-one (2.1 g) gave **UNT-TWU-8** as a dark red solid (1.3 g, 35% yield), m.p. 280-281 °C (decomposition begins at 225 °C). HRMS: $M^{+} + H$, 333.0346. Requires $M^{+} + H$, 333.0345.

Selected infrared absorptions (cm^{-1}): 3353, 3251, 3121, 2722, 1722, 1653, 1630 cm^{-1}

Characterization of this compound via proton NMR (^1H -NMR) was as follows:

^1H -NMR: δ 11.62 (s, 1H, NH), 8.74 (s, 1H, C(2)H), 8.18 [d, $J = 2.1$ Hz, 1H C(4)H], 7.39 [d, $J = 8.7$ Hz, 1H C(6)H], 7.26 [dd, $J = 8.4$ Hz and $J = 1.8$ Hz, 1H, C(7)H], 6.44 (s, 1H, C(8)H), 3.27 (s, 3H, N(2')CH₃), 3.08 (s, 3H, N(4')CH₃).

Synthesis of (E)-5-((5-chloro-1H-indol-3-yl)methylene)-2-imino-1,3-dimethylimidazolidin-4-one (UNT-TWU-9)

5-Chloroindole-3-carboxaldehyde (3.0 g) and 2-amino-1,3-dimethylimidazolidin-4-one (2.3 g) gave **UNT-TWU-9** (1.7 g, 60% yield), m.p. 245-246 °C (decomposition begins at 220 °C). HRMS: $M^{+} + H$, 289.0841. Requires $M^{+} + H$, 289.0851.

Synthesis of (E)-2-amino-5-((5-fluoro-1H-indol-3-yl)methylene)-1-methyl-1H-imidazol-4(5H)-one (UNT-TWU-10)

5-Fluoroindole-3-carboxaldehyde (1.0 g) and creatinine (0.75 g) gave **UNT-TWU-10**, (0.75 g, 75% yield), m.p. 291-292 °C (decomposition begins at 210 °C).

HRMS: $M^{+} + H$, 259.0992. Requires $M^{+} + H$, 259.099.

Selected infrared absorptions (cm^{-1}): 3375, 3266, 3151, 2729, 1650, 1621, 1564 cm^{-1}

Characterization of this compound via proton NMR (^1H -NMR) was as follows:

^1H -NMR: 9.15 [d, $J = 2.7$ Hz, 1H, C(2)H], 7.96 (s, DMF), 7.77 [dd, $J = 10.2$ and 2.4 Hz, 1H, C(4)H], 7.44 [dd, $J = 9.0$ and 4.7 Hz, 1H, C(7)H], 7.01 [td, $J = 9.0$ and 2.4 Hz, 1H, C(6)H], 3.29 (s, 3H, N(2)CH₃), 2.89 (s, DMF), 2.74 (s, DMF).

Synthesis of 5-((5-fluoro-1H-indol-3-yl)methylene)-2-imino-1,3-dimethyl-imidazolidin-4-one (UNT-TWU-11)

5-Fluoroindole-3-carboxaldehyde (1.0 g) and 2-imino-1,3-dimethyl-imidazolidin-4-one (0.93 g) gave **UNT-TWU-11** as orange crystals (0.85 g, 85%), m.p. 295-300 °C (decomposition begins at 245 °C). HRMS: $\text{M}^{+} + \text{H}$, 273.1149. Requires $\text{M}^{+} + \text{H}$, 273.1146.

Selected infrared absorptions (Nujol mull, cm^{-1}): 3357, 3311, 3132, 1722, 1651, 1632 cm^{-1} .

Characterization of this compound via proton NMR (^1H -NMR) was as follows:

^1H -NMR: δ 11.57 (s, 1H, NH), 8.76 [d, $J = 2.7$ Hz, 1H, C(2)H], 7.75 [dd, $J = 2$ Hz and $J = 10$ Hz, 1H, C(4)H], 7.42 [dd, $J = 9$ Hz and $J = 5$ Hz, 1H, C(7)H], 6.99 [tt, $J = 9$ Hz and $J = 2$ Hz, 1H, C(6)H], 6.64 (s, br, 1H, C=NH), 6.40 [s, 1H, C(8)H], 3.26 [s, 3H, N(2')CH₃], 3.07 [s, 3H, N(4')CH₃].

Synthesis of (E)-2-amino-5-((6-fluoro-1H-indol-3yl)methylene)-1-methyl-1H-imidazol-4(5H)-one (UNT-TWU-12)

6-Fluoroindole-3-carboxaldehyde (1.0 g) and creatinine (0.84 g) gave **UNT-TWU-12**. m.p. 258-260 °C (decomposition begins at 250 °C). HRMS: $M^{+} + H$, 259.0992. Requires $M^{+} + H$, 259.099.

Characterization of this compound via proton NMR (1H -NMR) was as follows:

1H -NMR: δ 11.49 [d, $J = 1.8$ Hz, 1H, NH], 9.06 [d, $J = 2.7$ Hz, 1H, C(2)H], 7.95 (s, DMF), 7.91 [dd, $J = 7.8$ and 6.3 Hz, 1H, C(4)H], 7.71 (s, br, 2H, NH_2), 7.21 [dd, $J = 9.8$ and 2.3 Hz, 1H, C(7)H], 6.97 [qd, $J = 3.0$, 2.4 and 0.9 Hz, 1H, C(5)H], 6.51 (s, 1H, C(8)H), 3.28 (s, 3H, N(2)CH₃), 2.89 (s, DMF), 2.73 (s, DMF).

Synthesis of (E)-5-((6-fluoro-1H-indol-3-yl)methylene)-2-imino-1,3-dimethylimidazolidin-4-one (UNT-TWU-13)

6-Fluoroindole-3-carboxaldehyde (0.50 g) and 2-amino-1,3-dimethylimidazolidin-4-one (0.46 g) gave **UNT-TWU-13**. (0.22 g, 44% yield) m.p. 260-264 °C (decomposition begins at 257 °C). HRMS: $M^{+} + H$, 273.1143. Requires $M^{+} + H$, 273.1146.

Selected infrared absorptions (cm⁻¹): 3359, 3124, 3072, 1721, 1650, 1633 1593 cm⁻¹

Characterization of this compound via proton NMR (¹H-NMR) was as follows:

¹H-NMR: δ 11.51 (s, 1H, NH), 8.68 [d, *J* = 2.1, 1H, C(2)H], 7.89, [dd, *J* = 9.0 and 5.7 Hz, 1H, C(7)H], 7.21 [dd, *J* = 9.0 and 2.4 Hz, 1H, C(4)H], 6.96 [td, *J* = 9.0, 2.3 and 1.8 Hz, 1H, C(5)H], 6.42 (s, 1H, C(8)H), 3.25 (s, 3H, N(4')CH₃), 3.06 (s, 3H, N(2')CH₃).

Synthesis of (E)-2-amino-5-((6-chloro-1H-indol-3-yl)methylene)-1-methyl-1H-imidazol-4(5H)-one (UNT-TWU-14)

6-Chloroindole-3-carboxaldehyde (1.0 g) and creatinine (0.68 g) gave **UNT-TWU-14**. (0.44 g, 44% yield). 300-302 °C (decomposition begins at 296 °C). HRMS: M⁺ + H, 289.085. Requires M⁺ + H, 289.0851.

Selected infrared absorptions (cm⁻¹): 3448, 3184, 3148, 3080, 1660, 1622 cm⁻¹

Characterization of this compound via proton NMR (¹H-NMR) was as follows:

¹H-NMR: δ 11.57 (s, 1H, NH), 9.10 [d, *J* = 1.8 Hz, 1H, C(2)H], 7.93 [d, *J* = 8.4 Hz, 1H, C(7)H], 7.75 (s, 2H, C-NH₂), 7.48 [d, *J* = 2.1 Hz, 1H, C(5)H], 7.12 [dd, *J* = 8.4 Hz and *J* = 1.8 Hz, 1H C(4)H], 3.28 (s, 3H, CH₃), 2.89 (s, DMF), 2.73 (s, DMF).

Synthesis of (E)-5-((6-chloro-1H-indol-3-yl)methylene)-2-imino-1,3-dimethylimidazolidin-4-one (UNT-TWU-15)

6-Chloroindole-3-carboxaldehyde (1.0 g) and 2-amino-1,3-dimethylimidazolidin-4-one (0.77 g) gave **UNT-TWU-15**. (0.74 g, 74% yield) m.p. 278- 280 °C

(decomposition begins at 276 °C). HRMS: $M^{+} + H$, 289.0851. Requires $M^{+} + H$, 289.0851

Selected infrared absorptions (cm^{-1}): 3340, 3128, 3063, 1723, 1646, 1632 cm^{-1}

Characterization of this compound via proton NMR (^1H -NMR) was as follows:

^1H -NMR: δ 11.57 (s, 1H, NH), 8.70 (s, 1H, C(2)H), 7.91 [d, $J = 8.7$ Hz, 1H C(6)H], 7.47 [d, $J = 1.8$, 1H C(5)H], 7.17 [dd, $J = 8.4$ Hz and $J = 1.5$, 1H, C(3)H], 6.75 (s, 1H, C-NH), 6.40 (s, 1H, C(8)H), 3.34 (s, 3H, N(4')CH₃), 3.25 (s, 3H, N(2')CH₃).

Synthesis of 5-((6-bromo-1H-indol-3-yl)methylene)-2-imino-1,3-dimethyl-imidazolidin-4-one (UNT-TWU-16)

6-Bromoindole-3-carboxaldehyde (1.0 g) and 2-imino-1,3-dimethyl-imidazolidin-4-one (**14**) (0.56 g) gave **UNT-TWU-16** as orange needles (0.55 g, 56% yield), m.p. 281-282 °C (decomposition begins at 269 °C). HR-MS: $M^{+} + H$, 333.0346. Requires $M^{+} + H$, 333.0345.

Selected infrared absorptions (Nujol mull, cm^{-1}): 3333, 3122 (w), 3065 (w), 1724, 1646, 1632 cm^{-1} .

Characterization of this compound via proton NMR (^1H -NMR) was as follows:

^1H -NMR: δ 11.58 (s, 1H, NH), 8.69 [s, 1H, C(2)H], 7.87 [d, $J = 9$ Hz, 1H, C(4)H], 7.61 [d, $J = 1.5$ Hz, 1H, C(7)H], 7.23 [dd, $J = 9$ Hz and $J = 1.5$ Hz, 1H, C(5)H], 6.75 and 6.54 (two s, br, 0.6H and 0.4H, C=NH), 6.39 [s, 1H, C(8)H], 3.25 [s, 3H, N(2')CH₃], 3.06 [s, 3H, N(4')CH₃].

Ultraviolet-visible spectrum: 381 nm (log ϵ = 4.50).

Synthesis of (Z)-3-ethyl-5-((6-fluoro-1H-indol-3-yl)methylene)-2-imino-1-methylimidazolidin-4-one (UNT-TWU-20)

6-Fluoroindole-3-carboxaldehyde (0.50 g) and 2-amino-3-ethyl-1-methylimidazolidin-4-one (0.43 g) gave **UNT-TWU-20** (0.27 g, 54% yield m.p. 330-331 °C (decomposition begins at 265 °C). HRMS: $M^{+} + H$, 287.1312. Requires $M^{+} + H$, 287.1303

Selected infrared absorptions (cm^{-1}): 3230, 3193, 3070, 2713, 1731, 1664, 1624 cm^{-1} .

Characterization of this compound via proton NMR (^1H -NMR) was as follows:

^1H -NMR: δ 12.08 (s, 1H, NH), 9.36 (s, 1H, C=NH), 8.97 [d, J = 2.7 Hz, 1H, C(2)H], 8.11 [dd, J = 9.0 and 5.4 Hz, 1H, C(4)H], 7.33 [dd, J = 9.0 and 3.2 Hz, 2H, C(8)H and C(7)H], 7.12 [td, J = 3.0, 2.4 and 2.1 Hz, 1H, C(5)H], 3.80 [q, J = 7.2 Hz, 2H, CH_2], 3.51 (s, 3H, N- CH_3], 1.19 [t, J = 6.0 Hz, 3H, CH_3].

Synthesis of (Z)-3-ethyl-2-imino-5-((5-iodo-1H-indol-3-yl)methylene)-1-methylimidazolidin-4-one (UNT-TWU-21)

5-Iodoindole-3-carboxaldehyde (0.50 g) and 2-amino-3-ethyl-1-methylimidazolidin-4-one (0.48 g) gave **UNT-TWU-21** (0.39 g, 78% yield), m.p. 290-292 °C. HRMS: $M^{+} + H$, 395.0385. Requires $M^{+} + H$, 395.0363.

Selected infrared absorptions (cm^{-1}): 3329, 3189, 2719, 1716, 1666, 1612 cm^{-1} .

Characterization of this compound via proton NMR (^1H -NMR) was as follows:

^1H -NMR: δ 12.15 (s, 1H, NH), 8.95 [d, $J = 2.7$ Hz, 1H, C(2)H], 8.53 (s, 1H, C=NH), 7.51 [dd, $J = 8.9$ Hz and $J = 2.0$ Hz, 1H, C(6)H], 7.36 [d, $J = 8.7$ Hz, 1H, C(7)H], 7.29 (s, 1H, C(8)H), 3.79 (q, $J = 6.9$ Hz, 2H, CH_2), 1.19 (t, $J = 6.9$ Hz, 3H, CH_3)

Synthesis of (E)-5-((5,6-dichloro-1H-indol-3-yl)methylene)-2-imino-1,3-dimethylimidazolidin-4-one (UNT-TWU-22)

5,6-Dichloroindole-3-carboxaldehyde (0.30 g) and 2-amino-1,3-dimethylimidazolidin-4-one (0.19 g) gave **UNT-TWU-22**, (0.15 g, 50% yield), m.p. 214 °C (dec). HSMS: $\text{M}^{++} + \text{H}$, 323.0462. Requires $\text{M}^{++} + \text{H}$, 323.0461.

Selected infrared absorptions (cm^{-1}): 3341, 2719, 1723, 1698, 1655, 1632 cm^{-1} .

Characterization of this compound via proton NMR (^1H -NMR) was as follows:

^1H -NMR: δ 11.7 (s, 1H, NH), 8.76, [d, $J = 1.5$ Hz, 1H, C(2)H], 8.28 (s, 1H, C(7)H), 7.95 (s, DMF), 7.68 (s, 1H, C(4)H), 6.41 (s, 1H, C(8)H), 3.26 (s, 3H, N(2') CH_3), 3.05 (s, 3H, N(4')H), 2.89 (s, DMF), 2.73 (s, DMF).

Synthesis of (Z)-5-((6-bromo-1H-indol-3yl)methylene)-3-ethyl-2-imino-1-methylimidazolidin-4-one (UNT-TWU-23)

6-Bromoindole-3-carboxaldehyde (0.50 g) and 2-amino-3-ethyl-1-methylimidazolidin-4-one (0.31 g) gave **UNT-TWU-23** (0.12 g, 24% yield), mp 310-315 °C (decomposition begins at 300 °C). HRMS: $M^{+} + H$, 347.0505. Requires $M^{+} + H$, 347.0502

Selected infrared absorptions (cm^{-1}): 3205, 3091, 2725, 1737, 1682, 1633 cm^{-1} .

Characterization of this compound via proton NMR (^1H -NMR) was as follows:

^1H -NMR: δ 12.11 (s, 1H, NH), 9.37 (s, 1H, C=NH), 8.97 [d, $J = 2.7$, 1H, C(2)H], 8.07 [d, $J = 8.7$ Hz, 1H, C(7)H], 7.72 [d, $J = 0.9$, 1H, C(4)H], 7.37 [dd, $J = 8.4$ Hz and 1.5 Hz, 1H, C(5)H], 7.31 (s, 1H, C(8)H), 3.79 (m, $J = 7.2$ Hz, 2H, CH_2), 1.19 (t, $J = 7.05$, 3H, CH_3).

Synthesis of (Z)-5-((5-bromo-1H-indol-3yl)methylene)-3-ethyl-2-imino-1-methylimidazolidin-4-one (UNT-TWU-24)

5-Bromoindole-3-carboxaldehyde (0.37 g) and 2-amino-3-ethyl-1-methylimidazolidin-4-one (0.23 g) gave **UNT-TWU-24** (0.26 g, 70% yield), m.p. 305-307 °C (decomposition begins at 295 °C). HRMS: $M^{+} + H$, 347.0508. Requires $M^{+} + H$, 347.0502.

Selected infrared absorptions (cm^{-1}): 3318, 3194, 3148, 1716, 1666, 1612 cm^{-1} .

Synthesis of (E)-5-((1H-indol-3yl)methylene)-2-amino-1-methyl-1H-imidazol-4(5H)-one (UNT-TWU-26)

Indole-3-carboxaldehyde (2.0 g) and creatinine (1.4 g) gave **UNT-TWU-26** (1.1 g, 58% yield), m.p. 287-290 °C (decomposition begins at 250 °C). HRMS: $M^{+} + H$, 241.1087. Requires $M^{+} + H$, 241.1084.

Selected infrared absorptions (cm^{-1}): 3360, 3184, 2714, 1675, 1650, 1613 cm^{-1} .

Characterization of this compound via proton NMR (^1H -NMR) was as follows:

^1H -NMR: δ 11.47 (s, 1H, NH), 9.09 [d, $J = 2.7$, 1H, C(2)H], 7.89 [d, $J = 7.2$ Hz, 1H, C(7)H], 7.71 (s, 2H, C-NH₂), 7.58 [d, $J = 7.2$, 1H, C(4)H], 7.13 (m, C(5)H and C(6)H), 6.55 (s, 1H, C(8)H), 3.29 (s, 2H, N(2')CH₃). The NMR is identical to that reported by Djura and Faulkner²⁶.

Synthesis of (E)-5-((1H-indol-3yl)methylene)-2-imino-1,3-dimethylimidazolidin-4-one (UNT-TWU-27)

Indole-3-carboxaldehyde (0.50 g) and 2-amino-1,3-dimethylimidazolidin-4-one (0.43 g) gave **UNT-TWU-27** (0.25 g, 50% yield), m.p. 245-247 °C (decomposition begins at 240 °C). HRMS: $M^{+} + H$, 255.1246. Requires $M^{+} + H$, 255.124.

Selected infrared absorptions (cm^{-1}): 3417, 3303, 2729, 2662, 1712, 1634 cm^{-1} .

Characterization of this compound via proton NMR (^1H -NMR) was as follows:

$^1\text{H-NMR}$: δ 11.48, (s, 1H, NH), 8.70 [d, $J = 2.7$ Hz, 1H, C(2)H], 7.88 [d, $J = 7.2$ Hz, 1H, C(7)H], 7.42 [dd, $J = 7.3$ Hz and $J = 1.4$ Hz, 1H, C(4)H], 7.13 (m, C(5)H and C(6)H), 6.44 (s, 1H, C(8)H), 3.23 (s, 3H, N(4'')CH₃), 3.07 (s, 3H, N(2')CH₃). NMR is identical to that reported by Djura and Faulkner²⁶.

Synthesis of (Z)-5-((1H-indol-3yl)methylene)-3-ethyl-2-imino-1-methylimidazolidin-4-one (UNT-TWU-28)

Indole-3-carboxaldehyde (0.50 g) and 2-amino-3-ethyl-1-methylimidazolidin-4-one (0.97 g) gave **UNT-TWU-28** (0.10 g, 20% yield), m.p. 320-324 °C (decomposition begins at 240 °C). HRMS: $\text{M}^{++} + \text{H}$, 269.1406. Requires $\text{M}^{++} + \text{H}$, 269.1397

Selected infrared absorptions (cm^{-1}): 3450, 3238, 3189, 2725, 2662, 1729, 1663, 1662, 1546 cm^{-1} .

Characterization of this compound via proton NMR ($^1\text{H-NMR}$) was as follows:

$^1\text{H-NMR}$: δ 12.08 [d, $J = 2.4$ Hz, 1H, NH], 9.35, (s, 1H, C=NH), 9.00 [d, $J = 3$ Hz, 1H, C(2)H], 8.08 [dd, $J = 6.6$ Hz and $J = 2.5$, 1H, C(7)H], 7.52 [dd, $J = 6.3$ and $J = 2.4$, 1H, C(4)H], 7.35 (s, 1H, C(8)H), 7.25 (m, C(5)H and C(6)H), 3.8 (q, $J = 7.2$ Hz, 2H, CH₂), 1.20 (t, $J = 7.2$, 3H, CH₃).

Synthesis of (E)-5-(5-chloro-1-H-indol-3-yl)methylene)-3-methylimidazolidine-2,4-dione (Figure 10)

5-Chloroindole-3-carboxaldehyde (0.35 g) and 3-methylimidazolidine-2,4-dione (0.22 g) in piperidine (2mL) were heated at 110 °C for 4 hrs under a nitrogen atmosphere. The reaction was allowed to cool to room temperature, vacuum filtrated, and washed with ether to give a yellow solid. The product was recrystallized from acetone to give a light yellow solid. Yield: 0.130g, 37%. m.p. 290 °C (dec). HRMS: $M^{++} + H$, 275.0465. Requires $M^{++} + H$, 275.0456.

Selected infrared absorptions (Nujol mull, cm^{-1}): 3317, 3176, 2359, 1753, 1737, 1696, 1644 cm^{-1} .

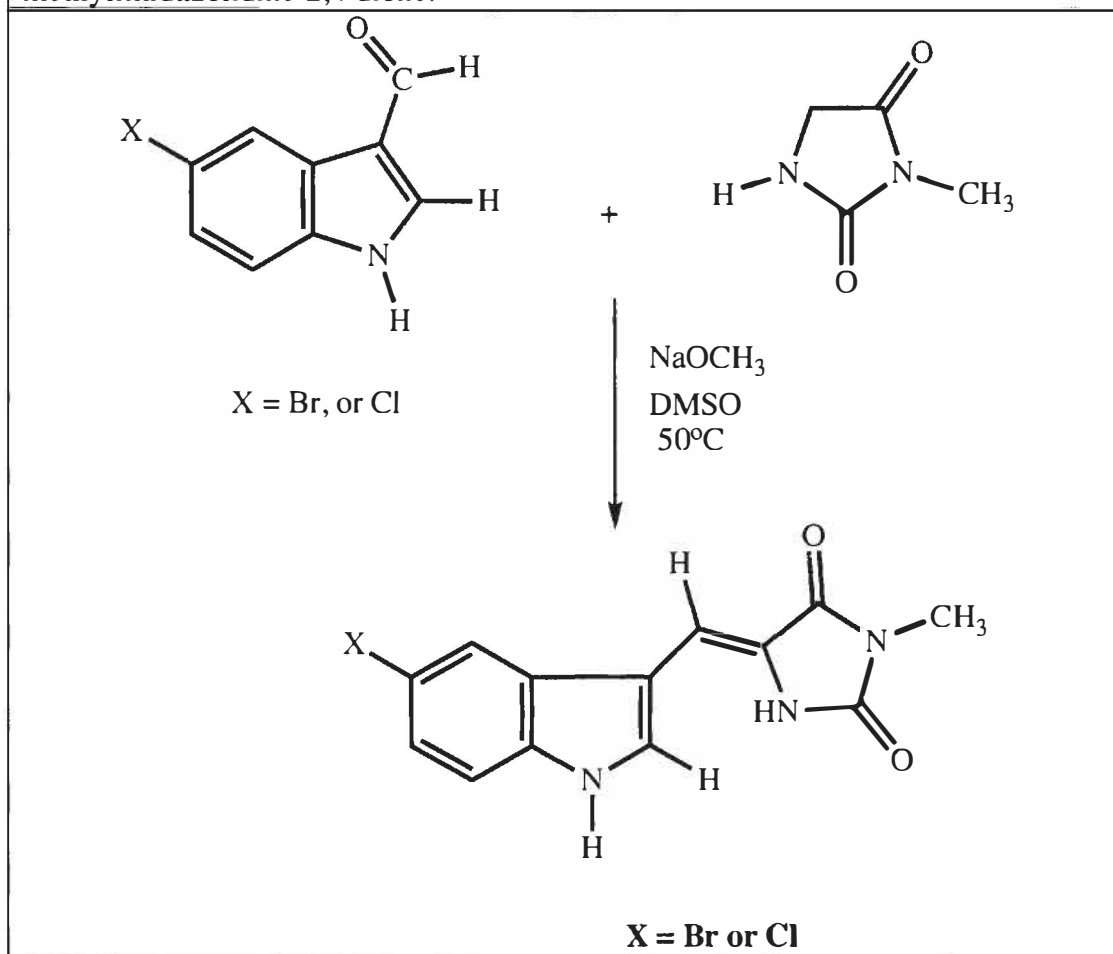
Characterization of this compound via proton NMR (^1H -NMR) was as follows:

^1H -NMR: δ 8.19 (s, 1H, C(2)H), 7.990 [d, $J = 2.1$ Hz, 1H, C(4)H], 7.44 [d, $J = 8.7$ Hz, 1H, C(7)H], 7.19 [dd, $J = 9.0$ and 1.95 Hz, 1H, C(6)H], 6.89 (s, 1H, C(8)H), 2.97 (s, 2H, N(4)CH₃).

Synthesis of (E)-5-((5-bromo-1H-indol-3-yl)methylene)-3-methylimidazolidine-2,4-dione (Figure 10)

A mixture of 5-bromoindolecarboxaldehyde (0.30 g), 3-methylimidazolidine-2,4-dione (0.23 g) and piperidine (2 mL) was refluxed for 4 h under a nitrogen atmosphere. The reaction was then cooled to room temperature, vacuum filtrated and rinsed with ether to give a bright yellow solid. The product was recrystallized from acetone to give a

Figure 10. Synthesis of 5-bromo or 5-chloro (1H-indol-3yl)methylene)-3-methylimidazolidine-2,4-dione.



bright yellow solid, (0.137 g, (46%)), m.p. 215-220 °C (dec). HRMS: $M^+ + H$, 318.9959.

Requires $M^+ + H$, 318.9951.

Selected infrared absorptions (Nujol mull, cm^{-1}): 3296, 3175, 3106, 2725, 2667, 1756, 1738, 1698, 1642.

Characterization of this compound via proton NMR (^1H -NMR) was as follows:

^1H -NMR: δ 8.17 (s, 1H, C(4)H), 8.04 [d, J = 1.5 Hz, 1H, C(4)H], 7.40 [d, J = 8.7 Hz, 1H, C(7)H], 7.31 [dd, J = 9.0 and 1.95 Hz, 1H, C(6)H], 6.89 (s, 1H, C(8)H), 2.97 (s, 3H, N(3)CH₃).

Synthesis of (Z)-5-(5-bromo-1H-indol-3yl)methylene)-3-methyl-2-thioxoimidazolidin-4-one

5-bromoindole-3-carboxaldehyde (1.5 g) was added to a solution of methyl-acetylthiohydantoin (1.0 g) in DMSO (2.0 mL) and sodium methoxide (0.0058 mol) in methanol (2.0 mL). The solution was heated to 50°C for 4 h and then cooled to room temperature. The solution was extracted with a saturated solution of sodium chloride and then with ether (3 x 50 mL). The ether layer was dried over anhydrous magnesium sulfate and rotary evaporated. The residue was recrystallized from acetone to give an orange/brown solid (0.034 g, 42%), m.p. 204–205 °C (dec). HRMS: $\text{M}^+ + \text{H}$, 334.9717. Requires $\text{M}^+ + \text{H}$, 334.9722.

Selected infrared absorptions (Nujol mull, cm^{-1}): 3303, 3201, 2714, 1856, 1749, 1722, 1695, 1635 cm^{-1} .

Characterization of this compound via proton NMR (^1H -NMR) was as follows:

^1H -NMR: 12.29 (s, 1H, N(3)H), 12.08 (s, 1H, NH), 8.57 [d, J = 2.4 Hz, 1H, C(2)H], 8.18 [d, J = 1.8 Hz, 1H, C(6)H], 7.50 [d, J = 8.4 Hz, 1H, C(7)H], 7.40 [dd, J = 6.0 and 1.8 Hz, 1H, C(6)H], 7.04 (s, 1H, C(8)H), 3.27 (s, 3H, N(3)CH₃).

Binding Procedures

Preparation of Crude Membranes for Binding Assays

Cell membranes from human embryonic kidney cells (HEK 293) stably expressing human 5-HT_{1A}, 5-HT_{2A} or 5-HT_{2C} receptors were prepared as described previously by Hu *et al*²¹. Cells expressing the desired receptor were detached using 10 mL of Dulbecco's phosphate buffered saline (D-PBS) without Ca²⁺ and Mg²⁺ and incubated for 15 min. After 15 min, the cells were pipetted into a 50 mL conical centrifugation tube, and the remainder of the tube volume was filled with Earle's balanced salts without sodium bicarbonate (EBSS) and pelleted by centrifugation in a Sorvall Legend RT at 800 rpm (700 xg) for 10 min. After centrifugation, the pellets, after decantation, were resuspended using 10 mL of buffer (5 mM MgCl₂, 5mM Tris pH 7.4 at 4 °C) and homogenized using 8 full strokes using a dounce glass homogenizer and then poured into a high speed centrifugation tube. The membranes were centrifuged at 13,000 rpm in the SA-600 rotor for 1 h. The membrane pellets were resuspended in 25 mL cold binding buffer (50 mM Tris, pH 4 °C) and washed by recentrifugation for an additional h. The resulting crude membrane pellets, after decantation, were resuspended in a small amount of cold binding buffer (1 data set = approximately 6 mL of 50 mM Tris, pH 7.4 at 4 °C), homogenized by 4 strokes of the dounce homogenizer, and kept in ice until ready to use the same day.

Radioligand Binding Assays

Single Point Binding Assay

Membranes, stably expressing cloned human 5-HT_{1A} (HEK 293, h5-HT_{1A}, #5, 9-21-06 CWC) receptors, were assayed for specific 4-(2-methoxy-)-phenyl-1-[2-(N-2-pyridyl)-*p*-fluorobenzamido]ethyl-piperazine ([³H]MPPF) (79.8 Ci/mmol) binding activity; cells expressing serotonin 5-HT_{2A} (HEK 293, h5-HT_{2A}, #4, 9-12-06. CWC) receptors were assayed for specific [³H]methylspiperone (84 Ci/mmol) binding activity; and cells expressing serotonin 5-HT_{2C} (HEK 293, h5-HT_{2C}, #23, 04-27-06, AAAC) receptors were assayed using [³H]mesulergine (80 Ci/mmol) for specific activity by saturation isotherm analysis as described by Hu *et al*²¹. Nonspecific binding activity was determined in the presence of 5 μM NAN-190 for 5-HT_{1A} and in the presence of 5 μM mianserin for 5-HT_{2A} and 5-HT_{2C}. For the single point binding assay, mirtazapine was also used as a reference (See Table 2 for drugs used in binding reaction). The reaction was kept for 1.5 h at 25 °C to reach equilibrium prior to rapid filtration through GF/C filters pretreated for at least 10 min with 0.5% polyethyleneimine. The filtered radioligand-receptor complexes were washed 3 times using ice-cold binding buffer (50 mM Tris pH 7.4 at 0 °C). Radioactivity bound to the filters was quantified by scintillation spectroscopy. Counts per minute were determined using a liquid scintillation counter (Perkin Elmer Tri-Carb 2800TR). Membrane protein concentrations were determined using the bicinchonic acid protein reagent (BCA) and a standard curve. Drug-binding affinity values were determined by inhibition curves. Inhibition constants

(K_i) for aplysinopsin analogs were determined by competition binding with the radioligands described above. The aplysinopsin analogs were prepared at an initial concentration of 3 mM in DMSO with the exception of the less soluble **UNT-TWU-16** that was prepared at a concentration of 1 mM in DMSO. The DMSO solutions were then diluted to 1:1000 v/v for the final assay solution.

Table 2. Receptor, radioligand and drugs for defining non-specific binding for testing UNT-TWU compounds. Each radioligand was used at 0.5 nM.

Receptor	Radioligand	Drug to define non-specific binding
5-HT2A (h5-HT2A, #4, 9-12-06, CWC)	[³ H] Methylspiperone a.k.a. [³ H]MSP	5 μM mianserin
5-HT2C (h5-HT2C, #23, 4-27-06, AAC)	[³ H]Mesulergine a.k.a [³ H]MES	5 μM mianserin
5-HT1A (h5-HT1A, #5, 9-21-06, CWC)	[³ H] MPPF	5 μM NAN-190
MPPF = 4-(2'-Methoxy)-phenyl-1-[2'-(N-2-pyridinyl)-p-fluorobenzamido]ethyl-piperazine		
MES = N-6-(methyl-3H) Mesulergine		

Inhibition Assay (Full Dose Response)

The membranes stably expressing cloned human 5-HT2A receptors and 5-HT2C receptors were prepared as described above (single point binding assay procedure). For the inhibition assays, stock solutions (6 mM) of **UNT-TWU-13**, **UNT-TWU-15**, **UNT-TWU-16**, **UNT-TWU-20**, and **UNT-TWU-22**, were prepared using the highest concentration possible using either DMSO or a solution of 95:5 DMSO: 0.01 N HCl.

The samples were then diluted in different concentrations (concentration between 1 nM to 10,000 nM) while not exceeding 1:300 v/v ratio of non-aqueous solvent.

Calculations and Data Analysis

All points were run in triplicate and each experiment was repeated from three to four times, and averaged values were reported with their standard error. The inhibition constant (K_i) values for the UNT-TWU compounds were calculated from IC_{50} values using the Cheng-Prusoff equation: $K_i = IC_{50}/(1 + [ligand]/K_D)$. A 95% confidence interval was used for all curve-fitting procedures using Graphpad's Prism software.

CHAPTER III

RESULTS

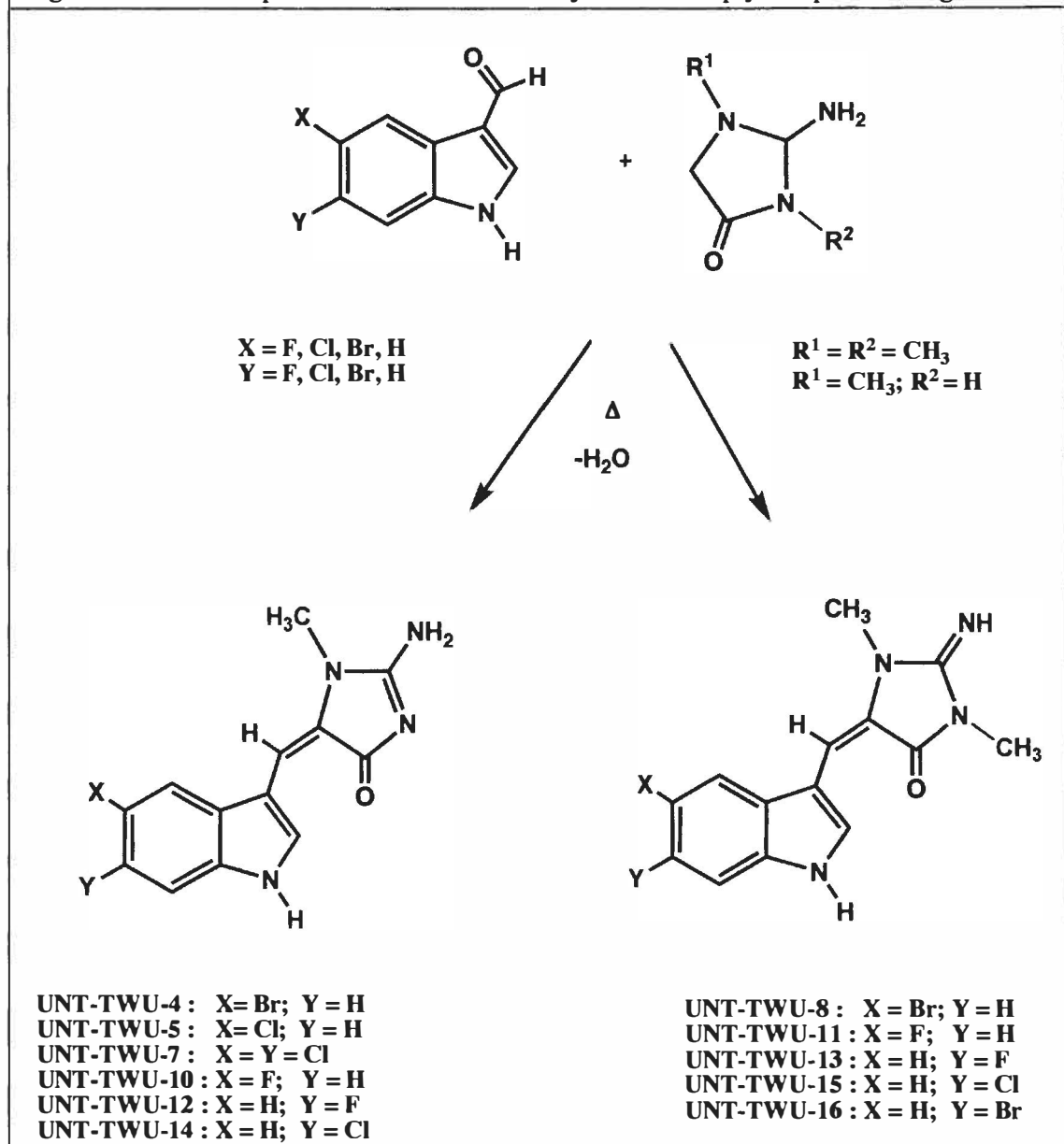
Synthesis

The successful synthesis of aplysinopsin analogs was accomplished in a manner similar to that described by Dujara and Faulkner²⁶ and as detailed in the methods section. Eleven aplysinopsin analogs were synthesized (Figure 11) and evaluated for their binding affinities.

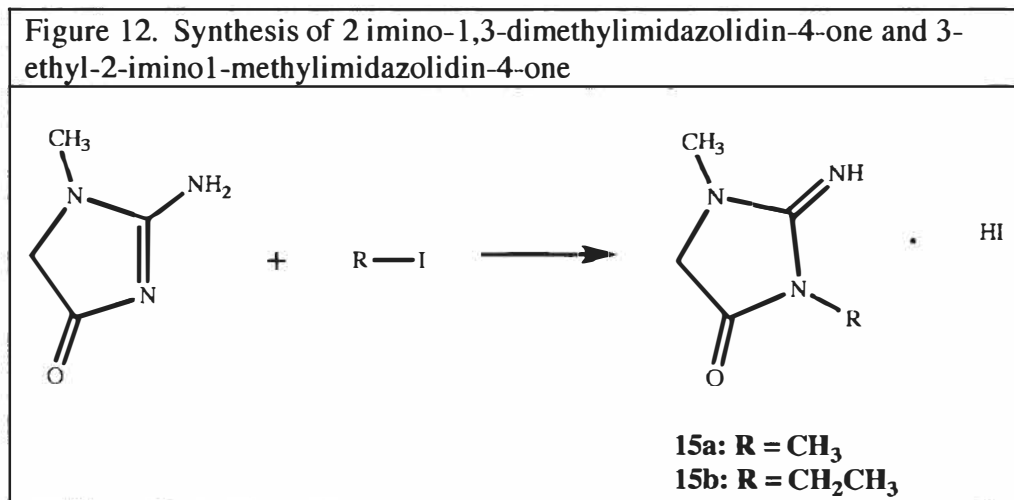
Figure 11 shows these eleven aplysinopsin analogs obtained by reacting the indole-3-carboxaldehyde with the alkylated creatinine. **UNT-TWU-5, -7, -10, -12, and -14** are monomethylated (one methyl group in the imidazolidine ring) compounds, while **UNT-TWU-8, -11, -13, -15, and -16** are compounds with two methyl groups in the imidazolidine ring,

The aldehydes needed for this research were either commercially available (5-bromoindole-3-carboxaldehyde and 5-chloroindole-3-carboxaldehyde) or were synthesized (Figure 6) using a procedure described by Jiang *et al*²³.

Figure 11. General procedure for the initial synthesis of aplysinopsin analogs



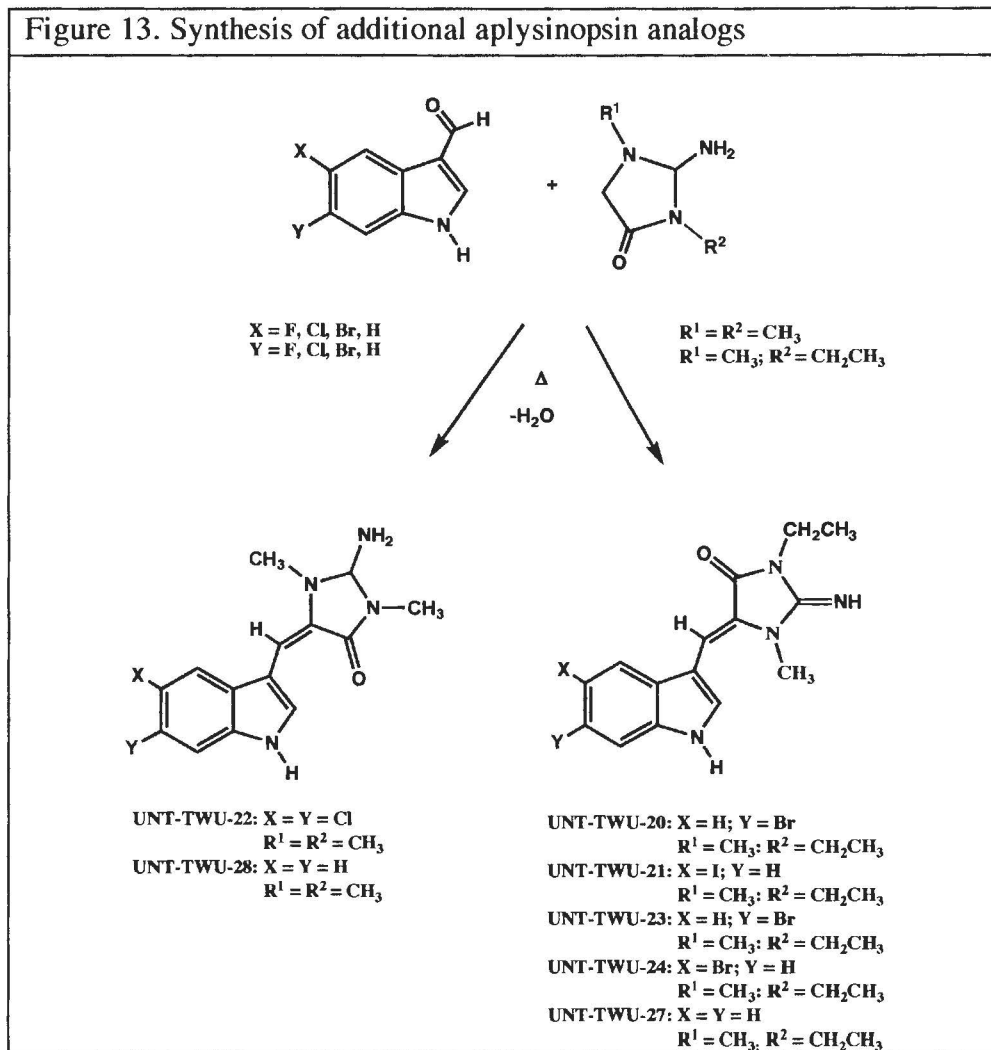
Creatinine was alkylated to give **(12a)** and **(12b)** using a procedure described by Kenyon *et al*²⁵. (Figure 12).



After initial binding results, additional compounds were synthesized as seen in Figure 13. The modification of these compounds was done on the imidazolidine ring. More specifically, a methyl group was exchanged for an ethyl group in an effort to improve their binding affinities. These seven compounds were also evaluated for their binding ability to displace specifically bound radioligands from 5-HT_{1A}, 5-HT_{2A} or 5-HT_{2C} receptors.

UNT-TWU-22 and **UNT-TWU-28** have 2 methyl groups in the imidazolidine ring, while **UNT-TWU-20**, **-21**, **-23**, **-24** and **-27** have a methyl group (CH₃) and ethyl group (-CH₂CH₃) in the imidazolidine ring.

Figure 13. Synthesis of additional aplysinopsin analogs



The single crystal structure was determined for two of the aplysinopsins (**UNT-TWU-4** and **UNT-TWU-11**) (Figures 14 and 15) synthesized in this project²⁷. A single crystal structure for 6-bromoindole-3-carboxaldehyde (Figure 16) was also obtained. The 6-bromoindole-3-carboxaldehyde was used for the synthesis of the 6-bromoaplysinopsin. Figure 14 shows the Oak Ridge Thermal Ellipsoid Plot (ORTEP) drawing of **UNT-TWU-4**, with 50% ellipsoids, showing the numbering scheme. Figure 15 shows the

ORTEP drawing of UNT-TWU-11, with 50% ellipsoids, and Figure 16 shows the ORTEP drawing of 6-bromoindole-3-carboxaldehyde, with 50% ellipsoids.

Figure 14. ORTEP drawing of UNT-TWU-4, with 50% ellipsoids, showing the numbering scheme

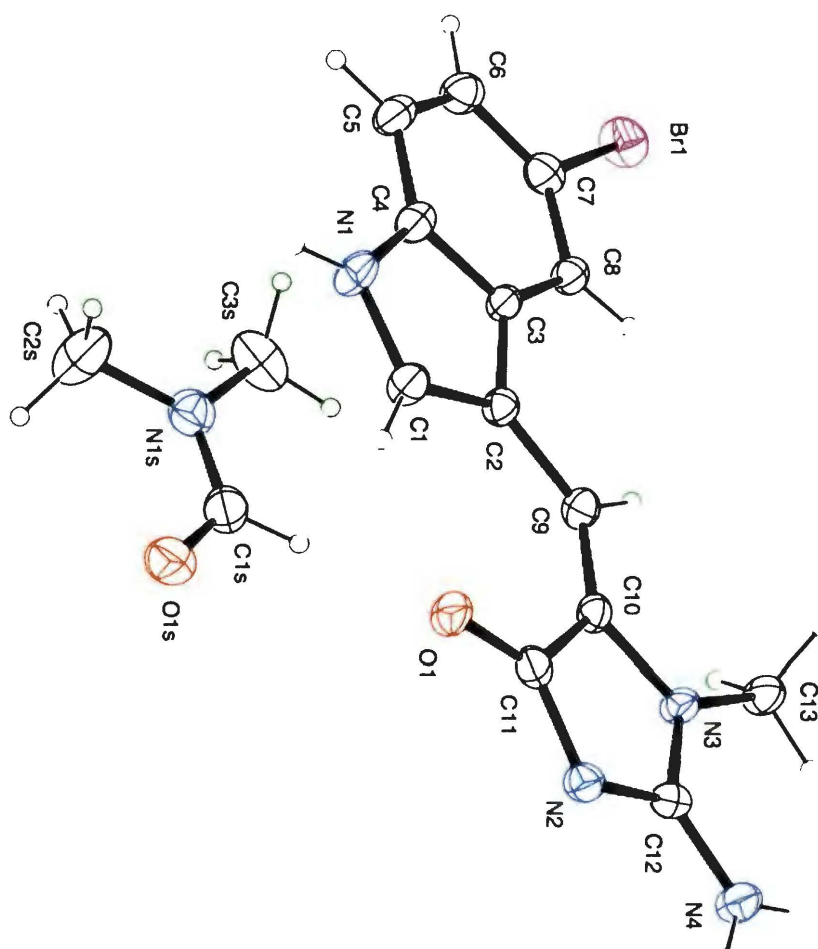


Figure 15. ORTEP drawing of UNT-TWU-11, with 50% ellipsoids

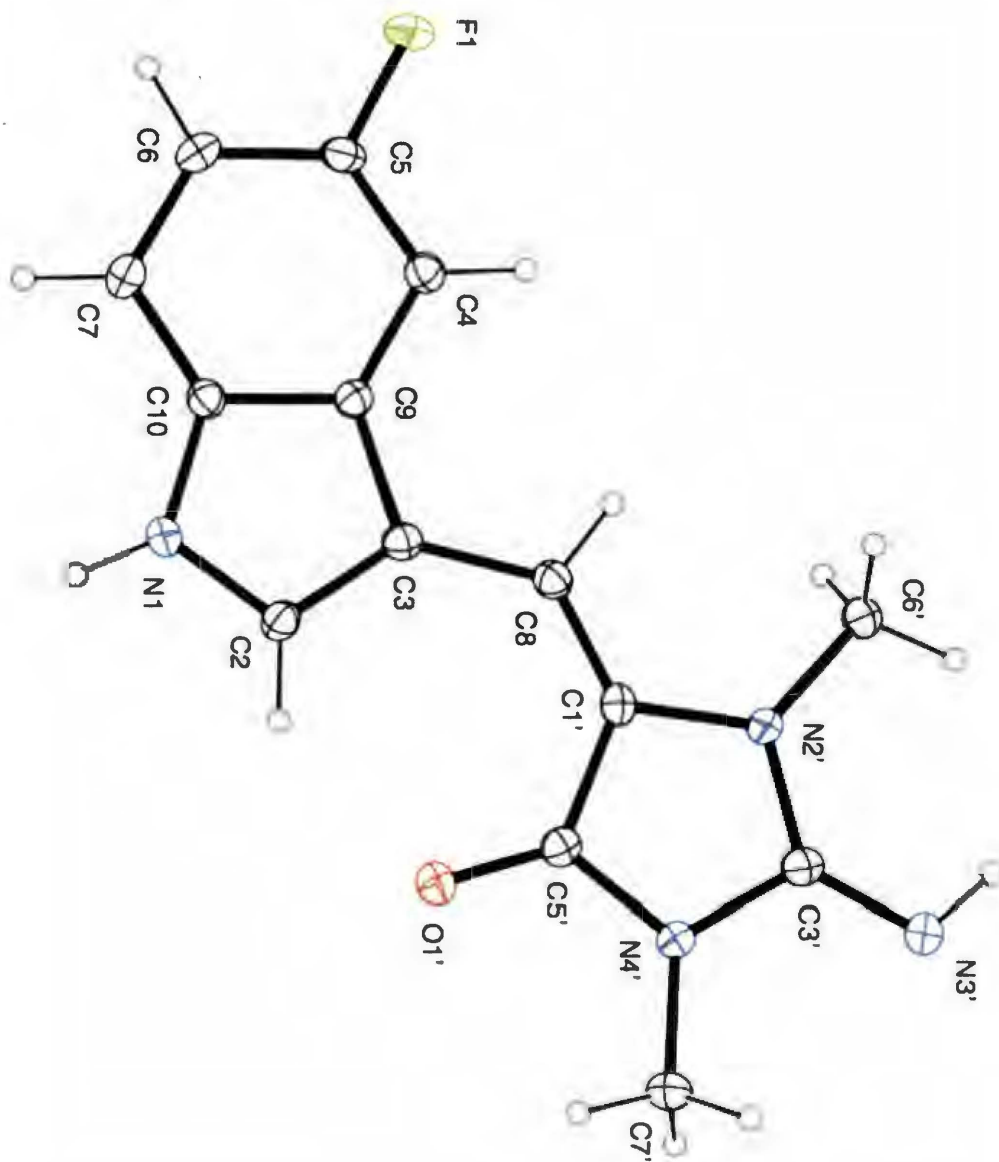
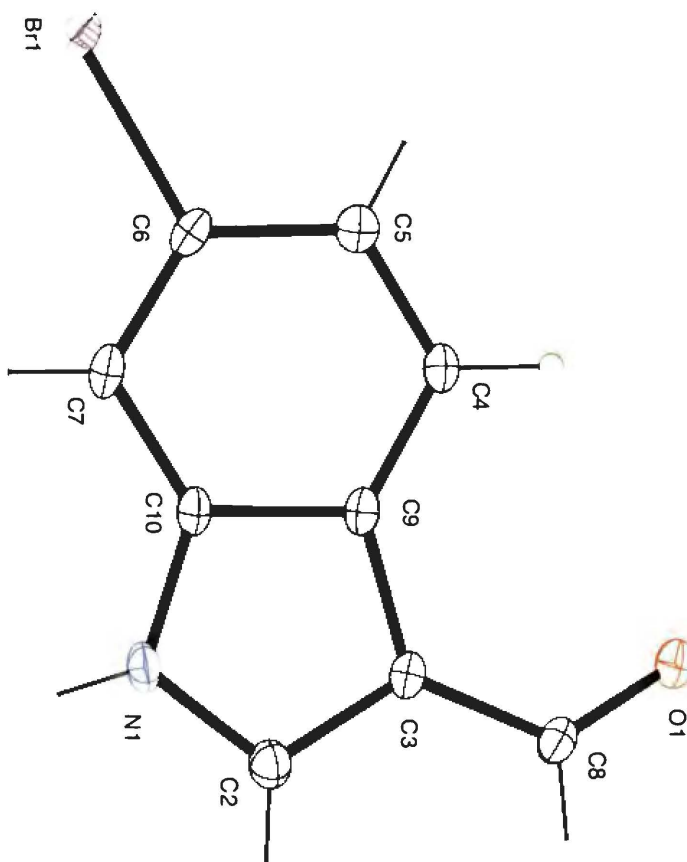


Figure 16. ORTEP drawing of 6-bromoindole-3-carboxaldehyde, with 50% ellipsoids



Binding Assays

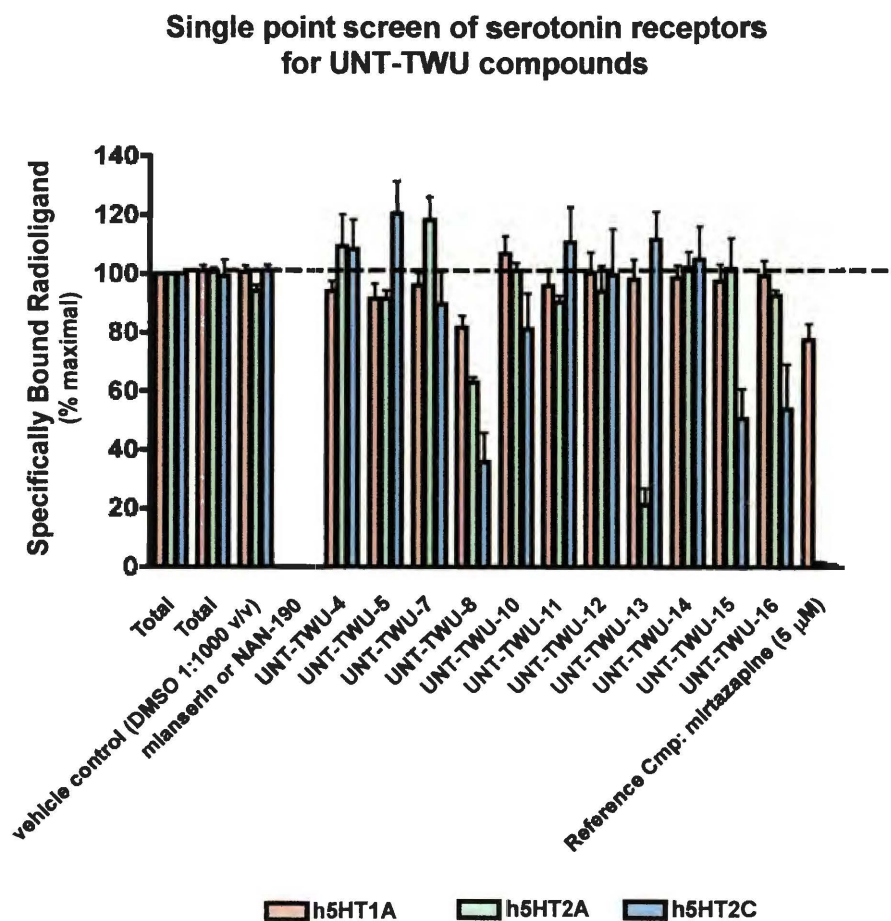
Initial Screening

A series of synthesized compounds containing the aplysinopsin pharmacophore were screened at a single concentration (1 – 3 μM). Single point assays for the

aplysinopsin analogs **UNT-TWU-4, -5, -7, -8, -10, -11, -12, -13, -14, -15, and -16** are shown in Figure 17. The aplysinopsin analogs were screened against 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors stably expressed in HEK293 cells. The radioligands [³H]MPPF, [³H]methylspiperone, [³H]mesulergine were utilized for 5-HT_{1A}, 5-HT_{2A} and 5-HT_{2C} receptors, respectively. Specific binding was set at 100 and the ability of the UNT-TWU compounds to compete for binding is shown for **UNT-TWU-4, -5, -7, -8, -10, -11, -12, -13, -14, -15 and -16**. This initial screening (Figure 17) revealed that only **UNT-TWU-8, UNT-TWU-13, UNT-TWU-15 and UNT-TWU-16** showed binding activity. **UNT-TWU-8, UNT-TWU-15 and UNT-TWU-16** showed binding activity for 5-HT_{2C} while **UNT-TWU-13** showed activity for 5-HT_{2A}. Note that none of the compounds with a single methyl group in the imidazolidine ring were effective competitors. Only the compounds that were methylated on both positions of the imidazolidine ring competed for binding to the 5-HT receptors.

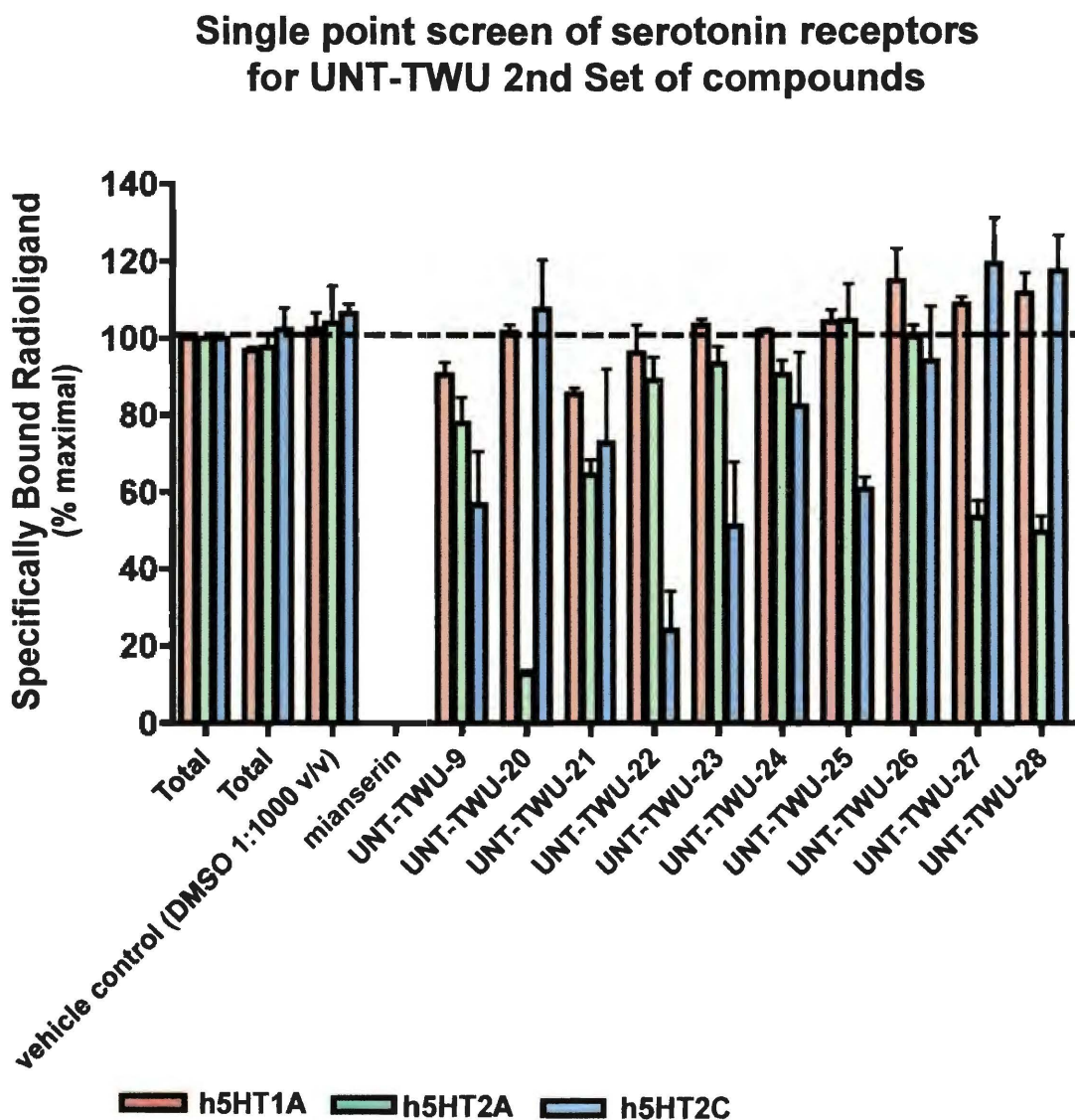
Therefore, modifications to our aplysinopsin analogs were made in expectation of increasing binding to 5-HT receptors. Single point assays for this second set of aplysinopsin analogs were performed as described above and the results are shown in Figure 18. From these data, it was observed that only **UNT-TWU-20, -22, -23, -27 and -28** showed significant binding activity. **UNT-TWU-20, -27 and -28** showed binding activity with 5-HT_{2A} receptors, while **UNT-TWU-22, -23 and -25** showed binding activity for 5-HT_{2C} receptors.

Figure 17. Single point screening of UNT-TWU compounds for cloned serotonin 5-HT1A, 5-HT2A, 5-HT2C receptors stably expressed in HEK293 cells. The radioligands [³H]MPPF, [³H]methyspiperone, [³H]mesulergine were utilized for 5-HT1A, 5-HT2A and 5-HT2C receptors, respectively. (Mean radioligand binding \pm SEM).



Note: All UNT-TWU compounds were tested at 3 μ M, except for UNT-TWU-16 which was tested at 1 μ M.

Figure 18. Single point screening of UNT-TWU compounds for cloned serotonin 5-HT1A, 5-HT2A, 5-HT2C receptors stably expressed in HEK293 cells. The radioligands [³H]MPPF, [³H]methylnspiperone, [³H]mesulergine were utilized for 5-HT1A, 5-HT2A and 5-HT2C receptors, respectively. (Mean radioligand binding \pm SEM).



Compound UNT-TWU-22 has two halogens in the indole ring (has a chlorine atom in the 5 and 6 position of the indole ring) and it is also dimethylated (2 methyl

groups in the imidazolidine ring). **UNT-TWU-23** is a 6-bromo compound (has a bromine in the 6th position of the indole ring) and has a methyl and an ethyl group in the imidazolidine ring; **UNT-TWU-27** has an ethyl and a methyl group in the imidazolidine ring but it has no halogens in the indole ring; and **UNT-TWU-28** has no halogen in the indole ring but has 2 methyl groups in the imidazolidine ring.

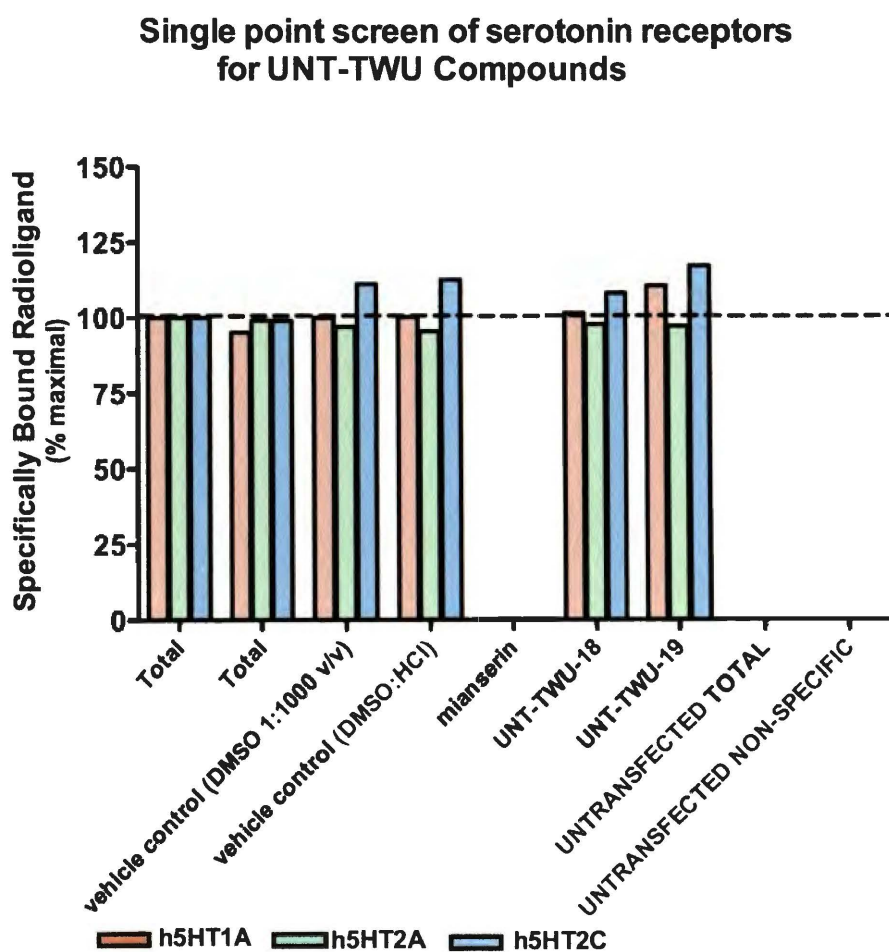
Figure 19 shows the compounds that did not bind to any of the receptors (5-HT1A, 5-HT2A and 5-HT2C). It is important to note that since these compounds did not show any binding ability to the receptors, only one experimental run was performed.

Full Dose Response Assays

UNT-TWU-13, UNT-TWU-15, UNT-TWU-16, UNT-TWU-20 and UNT-TWU-22 were chosen for full dose response curve testing (Figure 20, 21, 22, 23 and 24). These compounds showed some binding to 5-HT receptors after the single point assays. Membranes prepared for these cells were equilibrated with a fixed concentration of [³H]methylnaloxonium and [³H]mesulergine (~500 pM) and increasing concentrations of the competing compound. The amount of specifically bound radioligand following rapid filtration was plotted as a function of the logarithm of the concentration of the UNT-TWU compound. Nonspecific binding was defined by 5 μ M mianserin. Table 3 presents a compilation of UNT-TWU compounds that displayed binding affinity for all three receptors (5-HT1A, 5-HT2a and 5-HT2C receptors). Table 4 presents a compilation of UNT-TWU compounds that displayed binding affinity to either 5-HT2A or 5-HT2C

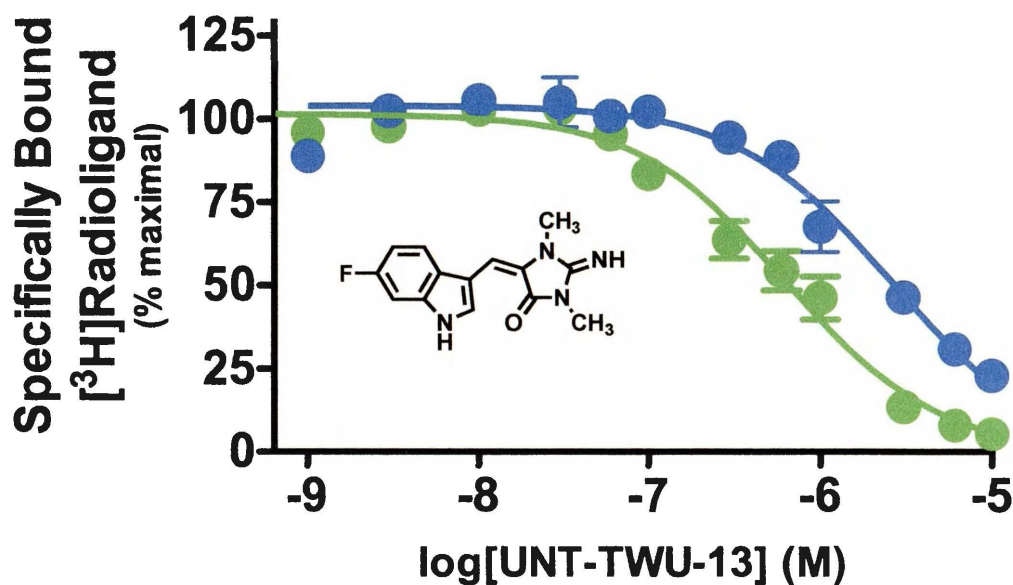
receptors. Table 5 presents a compilation of the activities of compounds **UNT-TWU-13**, **15**, **16**, **20** and **22**.

Figure 19. Single point screening of UNT-TWU compounds for cloned serotonin 5HT1A, 5HT2A, and 5HT2C receptors stably expressed in HEK293 cells. The radioligands [³H]MPPF, [³H]methylspiperone, [³H]mesulergine were utilized for 5-HT1A, 5-HT2A and 5-HT2C receptors, respectively. Since neither one of these UNT-TWU compounds were selective for either receptor, only one run was performed, therefore no error bars are present in this graph.



Note: All UNT-TWU compounds were tested at 3 μ M.

Figure 20. UNT-TWU-13 (see insert) inhibition of [³H]methylspiperone binding to 5-HT_{2A} receptors and UNT-TWU-13 inhibition of [³H]mesulergine binding to 5-HT_{2C} receptors. Membranes prepared for these cells were equilibrated with a fixed concentration of [³H]methylspiperone and [³H]mesulergine (~500 pM) and increasing concentrations of the competing compound (UNT-TWU-13). The amount of specifically bound [³H]methylspiperone following rapid filtration is plotted as a function of the logarithm of the concentration of UNT-TWU-13. Nonspecific binding was defined by 5 μ M mianserin. (Mean radioligand binding \pm SEM).



● 5HT2A

● 5HT2C

K_i (nM) \pm SEM

235 \pm 42

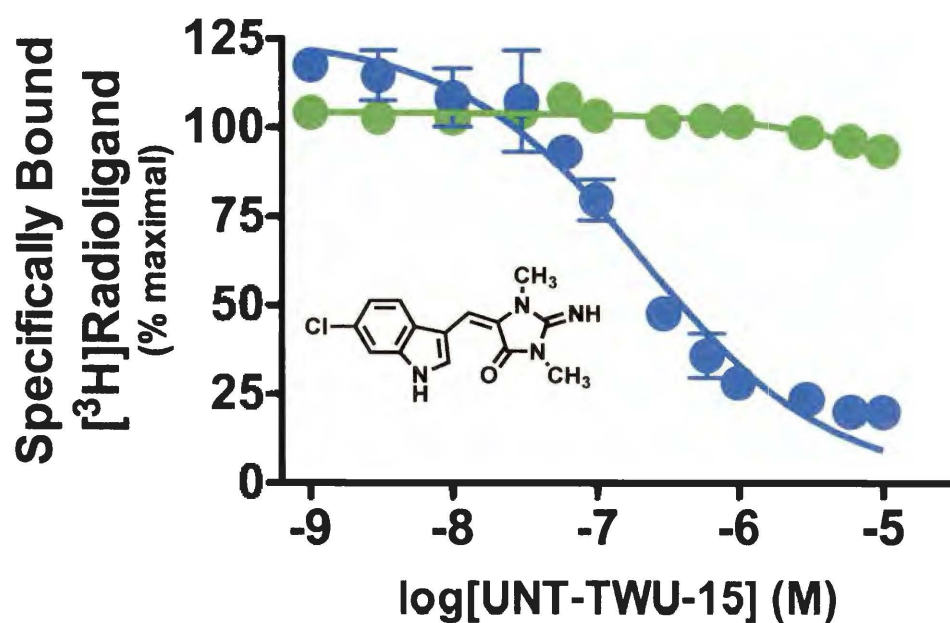
2114 \pm 203

IC₅₀

655

2562

Figure 21. UNT-TWU-15 (see insert) inhibition of [³H]methylspiperone binding to 5-HT_{2A} receptors and UNT-TWU-15 inhibition of [³H]mesulergine binding to 5-HT_{2C} receptors. Membranes prepared for these cells were equilibrated with a fixed concentration of [³H]methylspiperone and [³H]mesulergine (~500 pM) and increasing concentrations of the competing compound (UNT-TWU-15). The amount of specifically bound [³H]methylspiperone following rapid filtration is plotted as a function of the logarithm of the concentration of UNT-TWU-15. Nonspecific binding was defined by 5 μM mianserin. (Mean radioligand binding ± SEM).

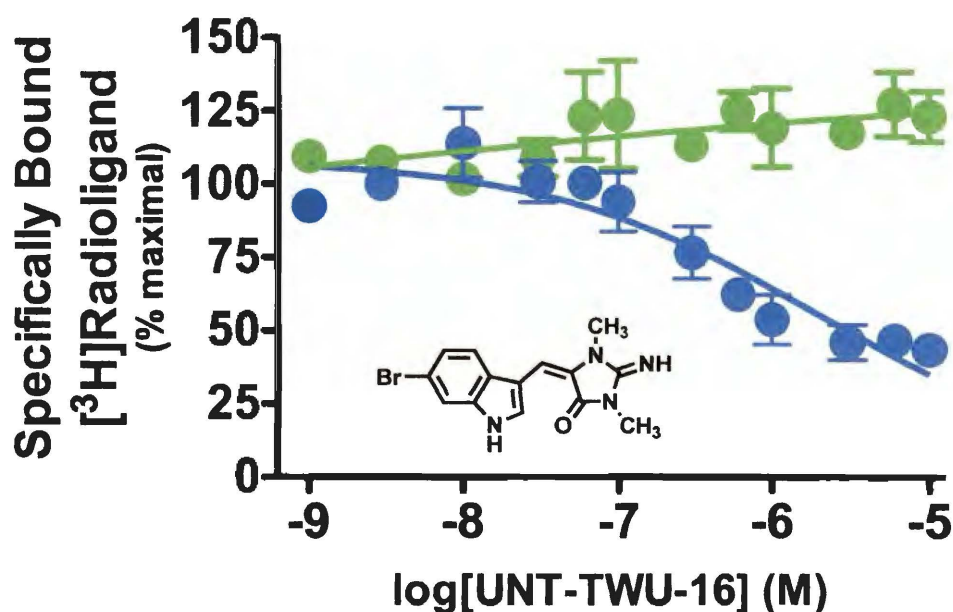


● 5HT_{2A} ● 5HT_{2C}

K_i (nM) ± SEM	N.D.	166 ± 55
IC ₅₀ (nM)	N.D.	309

N.D. not detected at highest concentration tested.

Figure 22. UNT-TWU-16 (see insert) inhibition of [³H]methylspiperone binding to 5-HT_{2A} receptors and UNT-TWU-16 inhibition of [³H]mesulergine binding to 5-HT_{2C} receptors. Membranes prepared for these cells were equilibrated with a fixed concentration of [³H]methylspiperone and [³H]mesulergine (~500 pM) and increasing concentrations of the competing compound (UNT-TWU-16). The amount of specifically bound [³H]methylspiperone following rapid filtration is plotted as a function of the logarithm of the concentration of UNT-TWU-16. Nonspecific binding was defined by 5 μ M mianserin. (Mean radioligand binding \pm SEM).



● 5HT_{2A} ● 5HT_{2C}

K_i (nM) \pm SEM	N.D.	2202 \pm 674
IC ₅₀ (nM)	N.D.	2133

N.D. = Not detected at highest concentration tested.

Figure 23. UNT-TWU-20 (see insert) inhibition of [³H]methylspiperone binding to 5-HT_{2A} receptors and UNT-TWU-20 inhibition of [³H]mesulergine binding to 5-HT_{2C} receptors. Membranes prepared for these cells were equilibrated with a fixed concentration of [³H]methylspiperone and [³H]mesulergine (~500 pM) and increasing concentrations of the competing compound (UNT-TWU-20). The amount of specifically bound [³H]methylspiperone following rapid filtration is plotted as a function of the logarithm of the concentration of UNT-TWU-20. Nonspecific binding was defined by 5 μM mianserin. (Mean radioligand binding ± SEM).

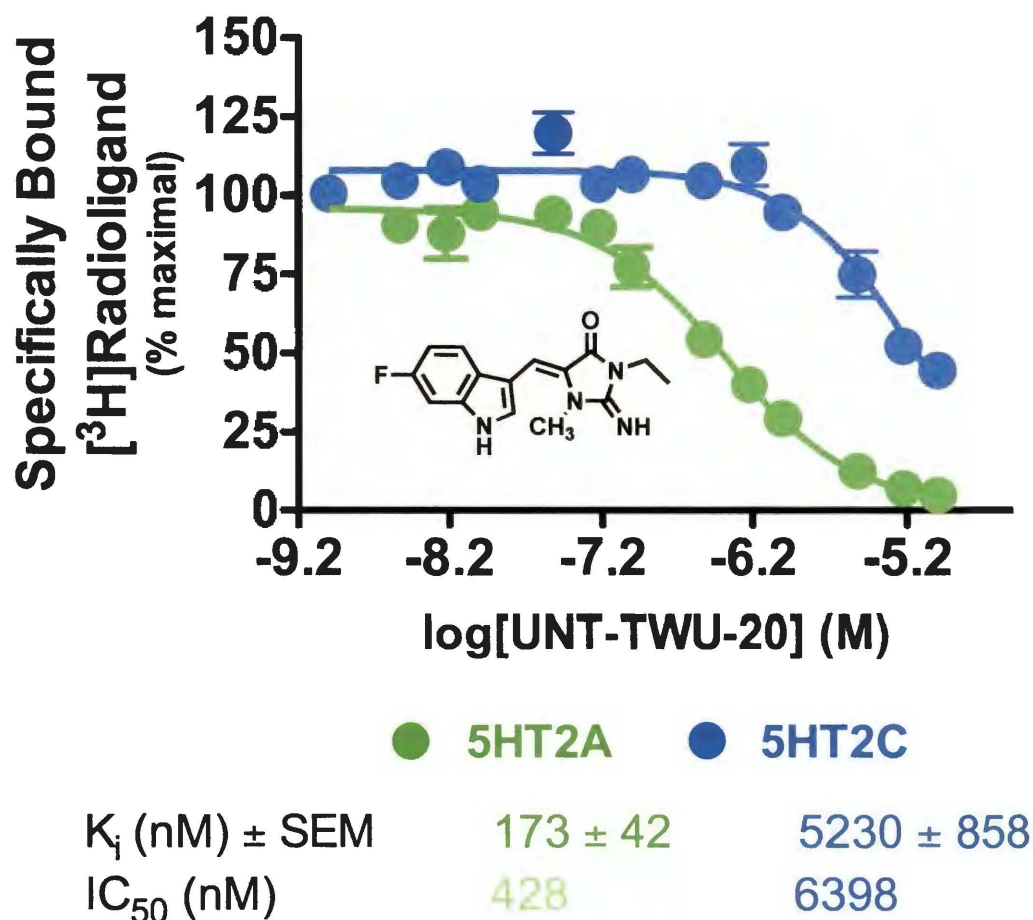
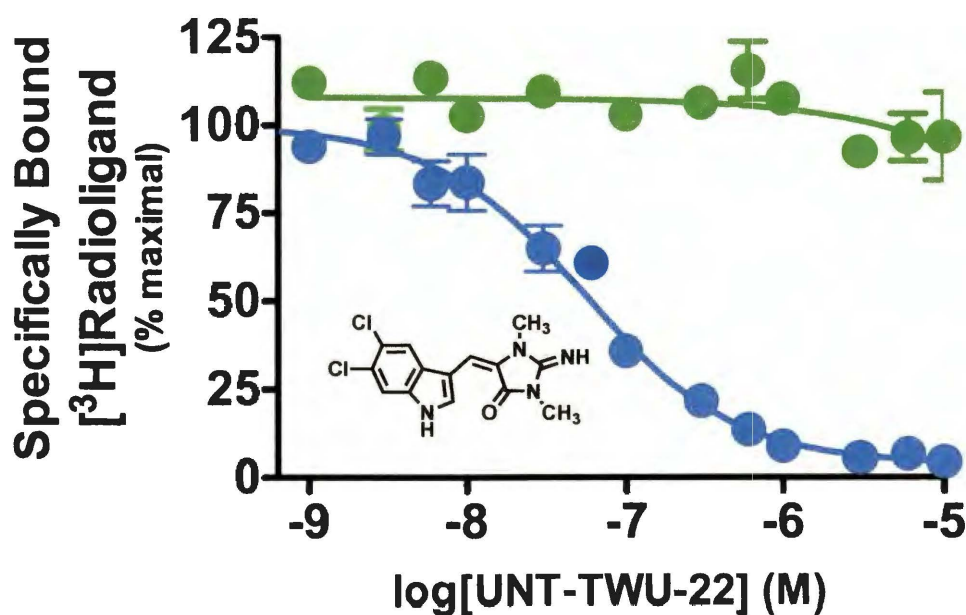


Figure 24. UNT-TWU-22 (see insert) inhibition of [³H]methylspiperone binding to 5-HT_{2A} receptors and UNT-TWU-22 inhibition of [³H]mesulergine binding to 5-HT_{2C} receptors. Membranes prepared for these cells were equilibrated with a fixed concentration of [³H]methylspiperone and [³H]mesulergine (~500 pM) and increasing concentrations of the competing compound (UNT-TWU-22). The amount of specifically bound [³H]methylspiperone following rapid filtration is plotted as a function of the logarithm of the concentration of UNT-TWU-22. Nonspecific binding was defined by 5 μM mianserin. (Mean radioligand binding ± SEM).



● 5HT_{2A} ● 5HT_{2C}

K_i (nM) ± SEM	N.D.	46 ± 8.6
IC_{50} (nM)	N.D.	N.D.

N.D. = Not detected at highest concentration tested.

With the exception of UNT-TWU-8, none of the monohalogenated aplysinopsin analogs showed binding affinity for 5-HT1A, 5-HT2A or 5-HT2C receptors. Also halogenation at the 6-position in the indole ring seems to control the binding selectivity of the aplysinopsin analogs towards 5-HT2A or 5-HT2C receptors. A fluorine at the 6-position showed selectivity for the 5-HT2A receptor while a Br or a Cl was selective for the 5-HT2C receptors.

Table 3. UNT-TWU compounds that displayed binding affinity for all three receptors (5-HT1A, 5-HT2A and 5-HT2C receptors)

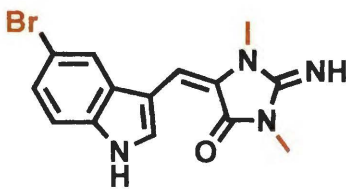
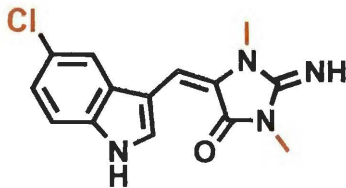
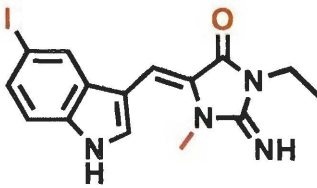
Affinity to 5-HT1A, 5-HT2A and 5-HT2C	
UNT-TWU-8	
UNT-TWU-9	
UNT-TWU21	

Table 4. UNT-TWU compounds that displayed binding affinity to either 5-HT2A or 5-HT2C receptors.

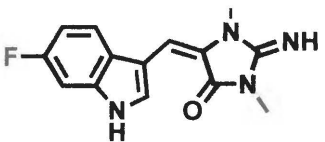
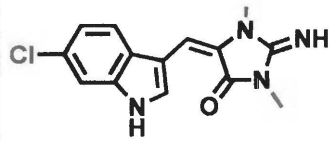
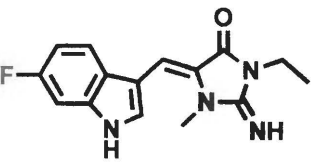
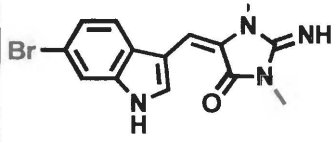
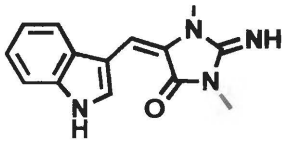
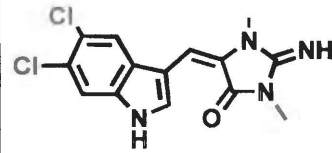
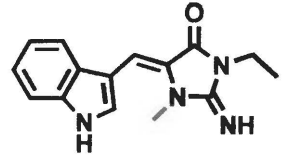
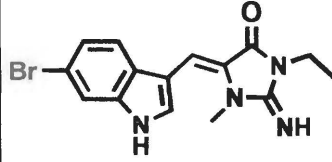
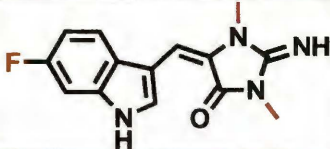
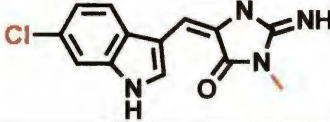
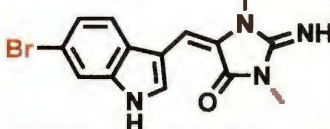
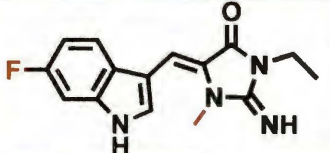
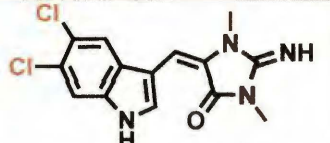
Compounds with affinity to 5HT2A		Compounds with affinity to 5HT2C	
Name	Structure	Name	Structure
UNT-TWU-13		UNT-TWU-15	
UNT-TWU-20		UNT-TWU-16	
UNT-TWU-27		UNT-TWU-22	
UNT-TWU-28		UNT-TWU-23	

Table 5. Affinity for cloned human serotonin 5-HT2 receptor subtypes.

Name	Structure	Binding Affinity (K_i nM)		K_i Selectivity Ratio *
		5-HT2A \pm SEM	5-HT2C \pm SEM	5-HT2A/5-HT2C
UNT-TWU-13		235 \pm 42	2114 \pm 203	0.11
UNT-TWU-15		N. D	166 \pm 55	>60
UNT-TWU-16		N.D	2202 \pm 674	>5
UNT-TWU-20		173 \pm 42	5230 \pm 858	0.033
UNT-TWU-22		N.D	46 \pm 8.6	>217

ND= not detected at highest concentration tested

* Assuming affinity is >10,000

CHAPTER IV

DISCUSSION

A naturally occurring compound called aplysinopsin was selected as the starting model for the twenty-two compounds synthesized in this project. This model was based upon the previous work of Hu *et al*²¹. demonstrating that aplysinopsin, purified from the natural product, has selectivity between the 5-HT_{2A} and the 5-HT_{2C} receptor subtypes.

In this study, we hypothesized that the affinity and selectivity for the human 5-HT_{2C} receptor subtype would be enhanced in synthetic aplysinopsin derivatives with halogen atoms on the 6 position of the indole ring and a methyl group on the 4' position of the imidazolidine ring system.

Synthesis Work

Synthesis was carried out by an aldol condensation of indole 3-carboxaldehydes with creatinine and creatinine derivatives. This procedure was first reported by Djura and Faulkner²⁶. This synthesis was attractive since creatinine is commercially available and the indole 3-carboxaldehydes are either commercially available or can be prepared easily from substituted indoles using a procedure described by Jiang *et al*.²³

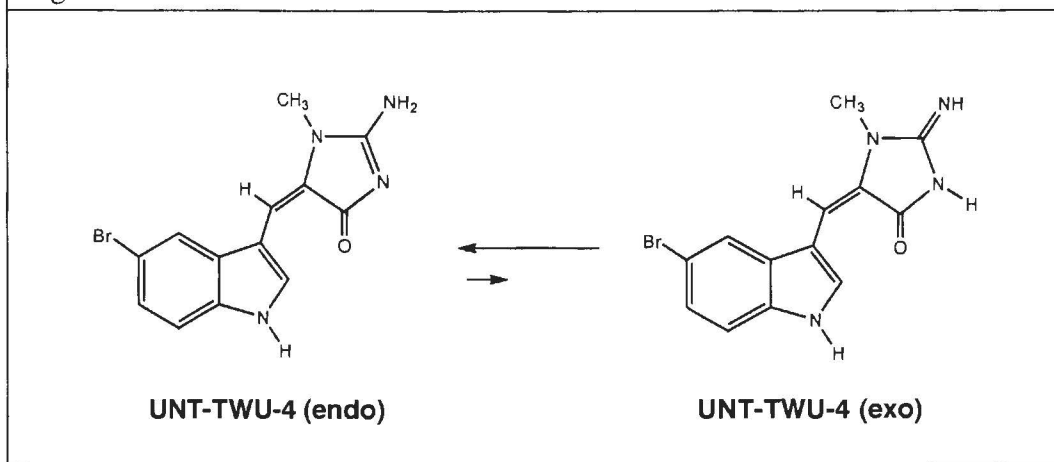
Out of the twenty-two compounds that were synthesized, only two of them had suitable crystals for single crystal X-ray structure. Although the configurations of some aplysinopsins have been determined from heteronuclear coupling constants^{21, 23, 28}, the X-ray structures provide unambiguous assignment of the configuration about the carbon-carbon double bond linking the indole ring to the imidazolodine ring in the aplysinopsin analogs reported here. Crystals of **UNT-TWU-4** were obtained by crystallization from DMF. The X-ray structure shows that a molecule of DMF is incorporated into the crystal lattice in a 1:1 molar ratio of DMF to aplysinopsin (Figure 14).

Compound **UNT-TWU-4** was synthesized from the reaction of 5-bromoindole-3-carboxaldehyde with creatinine in an acetic mixture containing sodium acetate salt³⁶. This compound was isolated from the reaction as the acetate salt and was not further purified.

The following was learned from the X-ray crystal structure determination:

1. The carbon-carbon double bond connecting the indole ring with the imidazolodine ring was determined to be in the *E* configuration.
2. The imidazol-4-one ring can exist in two different tautomeric forms, one where the imine double bond is endocyclic (**UNT-TWU-4**-endo (within the ring)) and the other where the imine double bond is exocyclic (**UNT-TWU-4**-exo (outside the ring)) (Figure 25).

Figure 25. Two different tautomeric forms for UNT-TWU-4



The crystal structure shows that the imidazolone ring exists in the endocyclic tautomeric form in the solid state. This was also true in solution since the ¹H-NMR shows absorption at 7.73 ppm integrating for two hydrogens representative of the NH₂ group. This indicates that this compound also exists in the endocyclic tautomeric form in d₆-DMSO solution. While the aplysinopsin analogs **UNT-TWU-8, 9, 11, 13, 15, 16, 20, 21, 22, 23, 24, 27** and **28** have an alkyl group on the imidazolone nitrogen atoms and therefore must exist in the exocyclic imine form, the aplysinopsin analogs, **UNT-TWU-4, 5, 7, 10, 12, 14, 17, and 26** are capable of existing in either form.

As stated earlier, the crystal structure of this compound shows the compound exists in the *E* isomer configuration; however, some naturally occurring aplysinopsins exist as a mixture of the *E* and *Z* isomers (Figure 26 and 27)²⁹. It is not clear from the literature on aplysinopsins whether the two isomers can be separated and isolated. There

are reports of mixtures of the *E* and *Z* isomers of aplysinopsins, including samples in which the aplysinopsin is reported to exist in a 50% mixture of each isomer²⁹. There is a report of an aplysinopsin that was mainly in the *Z* configuration [*Z/E* > 95/5] that was photoisomerized at 350 nm to give a mixture enriched in the *E* isomer²⁷. When this mixture was kept at room temperature in the dark for a few days, it isomerized back to the *Z* isomer.

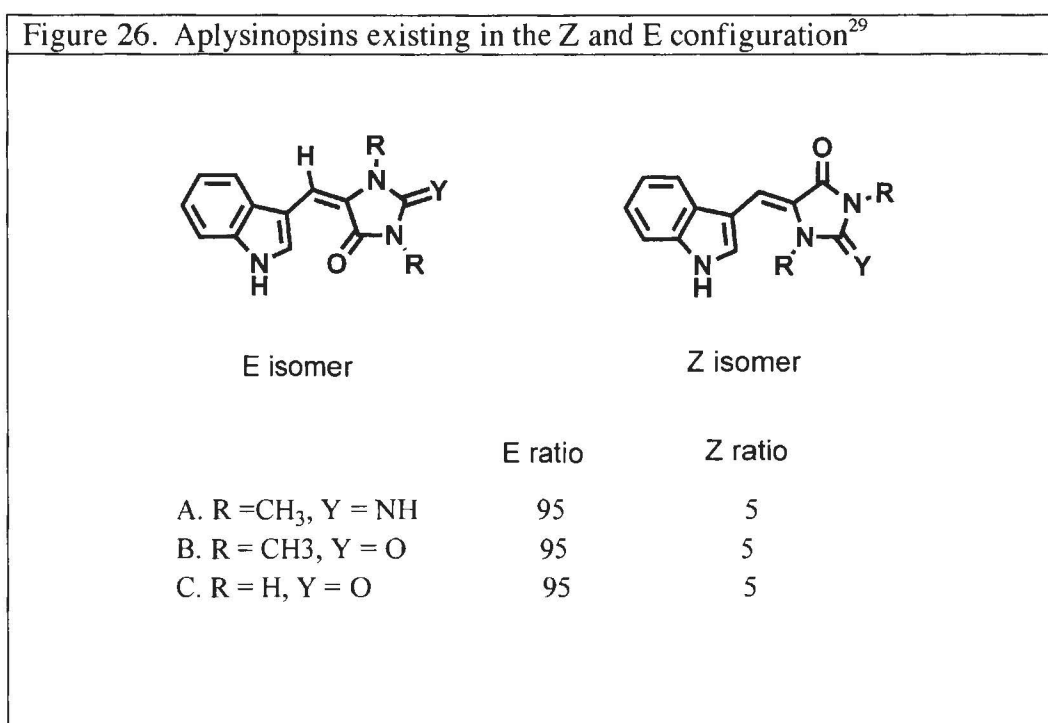
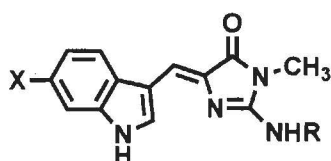
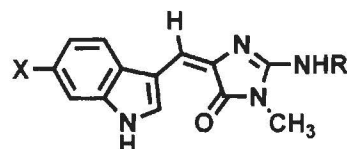


Figure 27. Aplysinopsins existing mainly in the *Z* configuration²⁹



Z isomer

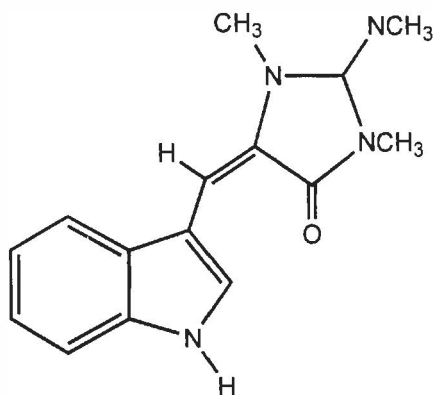


E isomer

	Z ratio	E ratio
D. X = R = H	95	5
E. X = Br, R = H	95	5
F. X = H, R = CH ₃	95	5
G. X = Br, R = CH ₃	95	5

It has been suggested that synthetic analogs and naturally occurring aplysinopsins without an alkyl group in the N(2') exist mainly in the *Z* configuration, while aplysinopsins with an alkyl group in the N(2') are mainly in the *E* configuration²⁸. In a review by Wells³⁰, it was reported that the X-ray structures for the *Z* and *E* isomers of methylaplysinopsin (Figure 28) have been carried out. However, the reference for this work is to work that, to our knowledge, has never appeared in print.

Figure 28. Methylaplysinopsin

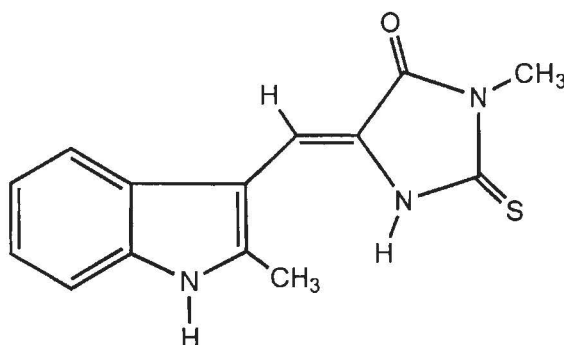


Methylaplysinopsin

Crystals of **UNT-TWU-11** were obtained by crystallization from DMF. An X-ray crystal structure was obtained. Once again the crystal structure shows the incorporation of a molecule of DMF in the crystal lattice. The X-ray structure of **UNT-TWU-11** is shown in Figure 15, but it does not include the DMF solvent molecule.

The only X-ray structure of an aplysinopsin or aplysinopsin analogs that has been reported was carried out on a thioaplysinopsin (Figure 29).

Figure 29. Thioaplysinopsin



Thioaplysinopsin

The X-ray structure of the thioaplysinopsin shows that this aplysinopsin analog is in the Z configuration³¹.

The crystal structure of 6-bromoindole-3-carboxaldehyde was also obtained. 6-Bromoindole-3-carboxaldehyde is the indole precursor used for the synthesis of **UNT-TWU-16**. This aldehyde compound is found in marine sponges²¹. The X-ray structure of indole-3-carboxaldehyde, which lacks the bromine atom, has been reported³². There are also X-ray structures on two indole-3-carboxaldehydes with substituents on the benzene ring³¹.

Structure-Affinity Relationship for Aplysinopsin Derivatives

The starting point of this research was an aplysinopsin, purified from a naturally occurring source. Hu *et al*²¹. showed that an aplysinopsin pharmacophore demonstrated

selectivity between 5-HT_{2A} and 5-HT_{2C} receptors subtypes. Hu *et al*²¹. also found that the natural product 6-bromo-2'-de-N-methylaplysins had a low affinity for the 5-HT_{2A} receptor but had high selectivity for 5-HT_{2C} receptor subtype.

We were able to synthesize the 6-bromoaplysinsin (**UNT-TWU-16**) which is identical to one of the compounds studied by Hu *et al*²¹. By synthesizing this compound, we were able to compare our results to those results found by Hu *et al*²¹. We found that the purified naturally occurring compound, 6-bromoaplysinsin, and the synthetic compound (**UNT-TWU-16**) showed different affinity values for the screened receptors but both of these compounds showed selectivity for the 5-HT_{2C} receptor. The synthetic version of this compound (**UNT-TWU-16**) had a 6-fold lower affinity for the 5-HT_{2C} receptor than the natural compound and as much as >50-fold lower affinity for the 5-HT_{2A} receptor subtypes. Although the natural product was screened in COS-7 cell line transient expression system, the other assay conditions were the same. One of the possibilities for this difference in values could be that the product purified from the natural source contained impurities not found in the synthetic compound.

We found in this study that the aplysinsin pharmacophore could be used as a model for creating small molecules that can differentiate between 5-HT_{2A} and 5-HT_{2C} receptors subtypes. This pharmacophore can be modified either in the indole ring or the imidazolidine ring. Based on the modification, one can create a molecule more selective for one receptor versus the other. The kind of halogenation in the indole ring, position 6, controls the selectivity for 5-HT_{2A} or 5-HT_{2C} receptors. If there is a small halogen such

as fluorine in the 6 position of the indole ring, the molecule becomes more selective for the 5-HT_{2A} receptor than for 5-HT_{2C}. The same effect is present when there is no halogen in the indole ring. However, if there is a chlorine or bromine in the 6-position of the indole ring, the molecule becomes more selective for the 5-HT_{2C} receptor subtype. It is important to mention that there were compounds that had a halogen in the 5-position of the indole ring but none of these compounds displayed selectivity for either receptor subtype. Based on our results, the 6-chloro aplysinopsin analog displayed the highest selectivity for the 5-HT_{2C} receptor; so it was decided to add another chlorine to the molecule at position 5 to see if the new compound (**UNT-TWU-22**) would show a higher affinity for the receptor. The addition of the halogen to the molecule increased the affinity for 5-HT_{2C} more than 3-fold compared to that of the monohalogenated compound **UNT-TWU-15**.

Out of twenty-two compounds synthesized for this project, only the compounds that were alkylated in position R2 and R4 of the imidazolidine ring showed binding activity. If there was a methyl group in both positions, the compound bound to the receptors. Changing the alkyl group in position R2 to a larger group such as an ethyl increased the affinity of this compound for the receptor. Based on the naturally occurring compound, we knew that a methyl group in position R4 was necessary for the molecule to bind. Since we did not obtain aplysinopsins with only a methyl group in position R4, we could not determine whether both alkyl groups (position R2 and R4) are necessary for binding or if only the alkyl group in the R4 position is needed. We know that in the

natural product, the alkyl group is present in the R4 position but none of our synthetic compounds had an alkyl group in that position. We either have the alkyl group on both positions or in position R2. Our work showed that only the molecules that were alkylated in both positions bound to the receptors and that the size of the alkyl group determined affinity of the compound for the receptors.

The study by Hu *et al.*²¹ also showed that in order for the molecule to bind, it must contain a nitrogen with the ability to be protonated in position R3. Therefore, the alkyl group in position R2 and R4 become more important for this molecule to bind. When both positions are occupied by an alkyl group, the imine nitrogen in position R3 becomes available for protonation allowing this molecule to bind to the receptor. However, when position R2 is not occupied by an alkyl group, the protonation is more likely to take place in the nitrogen in that position instead of the nitrogen in position R3. This is a reasonable possibility because it is known from previous studies³² in other biogenic amine G protein-coupled receptors that an imine nitrogen in the imidazolidine ring, with the ability to be protonated, is required for binding. This study³² also showed that oxygen or sulfur-containing compounds without a basic nitrogen atom have no detectable affinity for any of the receptors tested.

Further studies are necessary to fully understand the major contributing factor in the ability for molecules to bind to the receptors. Is it the size and number of halogens in the indole ring that determines selectivity? Or is it the alkyl group in the imidazolidine ring that is the determining factor for the ability of the molecule to bind?

Summary

In conclusion, in this study, we obtained two X-ray crystal structures that helped in the determination of the characterization of these aplysinopsin analogs. The X-ray structures have established that the compounds synthesized in this work exist in the *E* configuration. The X-ray structure of **UNT-TWU-4** establishes that the carbon-nitrogen double bond in the imidazolone ring is in the endocyclic tautomeric form in the solid state. We used natural occurring aplysinopsins as models for the synthesis of small molecules that were used to test selectivity for the human serotonin 5-HT_{2A} or 5-HT_{2C} receptor subtypes. This model could be used as a chemical scaffold for synthesizing compounds selective for the human serotonin 5-HT_{2A} or 5-HT_{2C} receptor subtypes. This research also shows that the aplysinopsin pharmacophore can be uniquely tuned for selectivity of 5-HT_{2A} over 5-HT_{2C} through a halogen selectivity at the 6-position of the indole ring. Future work on these aplysinopsin pharmacophores are likely to produce selective compounds with even higher affinities. These compounds could potentially be used in a variety of medical applications.

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APPENDIX A
Failed Synthetic Attempts

APPENDIX A

Failed Synthetic Attempts

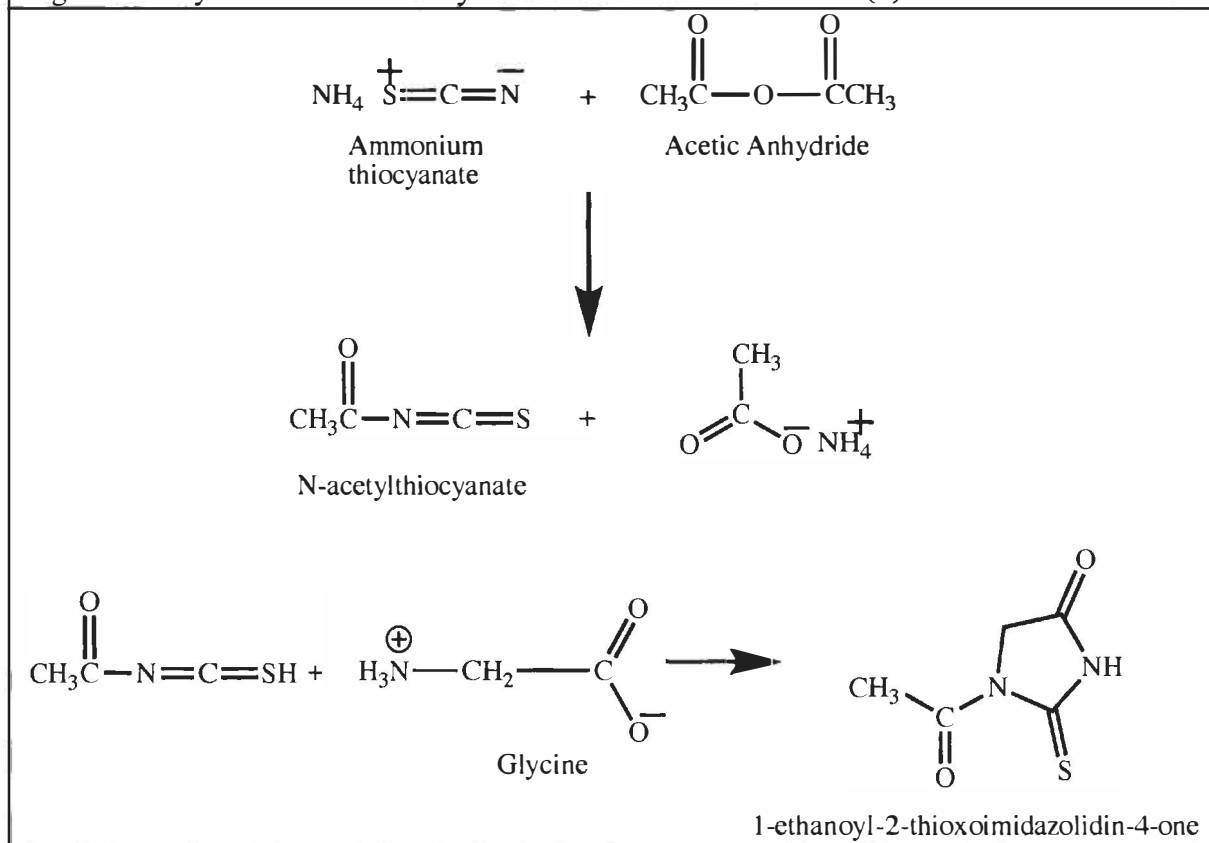
Synthesis of the desired project was initiated by an attempt to obtain the right part of the ring by using available starting materials and following reactions published with slight modifications.

The initial attempt to synthesize aplysinopsin analogs involved a multi-step synthesis of 5-((5-Bromo-1H-indol-3-yl)methylene)-3-methyl-2-thioxoimidazolidin-4-one.

Synthesis of 1-ethanoyl-2-thioxoimidazolidin-4-one (1) (Figure A1)

Acetic anhydride (100 mL) and ammonium thiocyanate (38.0 g) were mixed together and stirred for a couple of minutes; glycine (37.5 g) was added to the reaction mixture and the reaction was refluxed for 2 h. The reaction was cooled to room temperature and the solid was filtered using vacuum filtration and dried to yield a light brown solid; 7.68 g (21% yield) of product was recovered and the product had a melting point (m.p.) of 174-175 °C with decomposition. The reported m.p. of this product is 175 °C⁴.

Figure A1. Synthesis of 1-ethanoyl-2-thioxoimidazolidin-4-one (1)



Once the synthesis of 1-ethanoyl-2-thioxoimidazolidin-4-one was completed, it was necessary to synthesize the second required starting materials – the N-nitroso-N-methylurea (2). It is necessary to point out that most of the starting materials were not commercially available and thus created the need to synthesize most of them.

Synthesis of N-nitroso-N-methylurea (urea, α -methyl- α -nitroso) from methylamine hydrochloride (2)

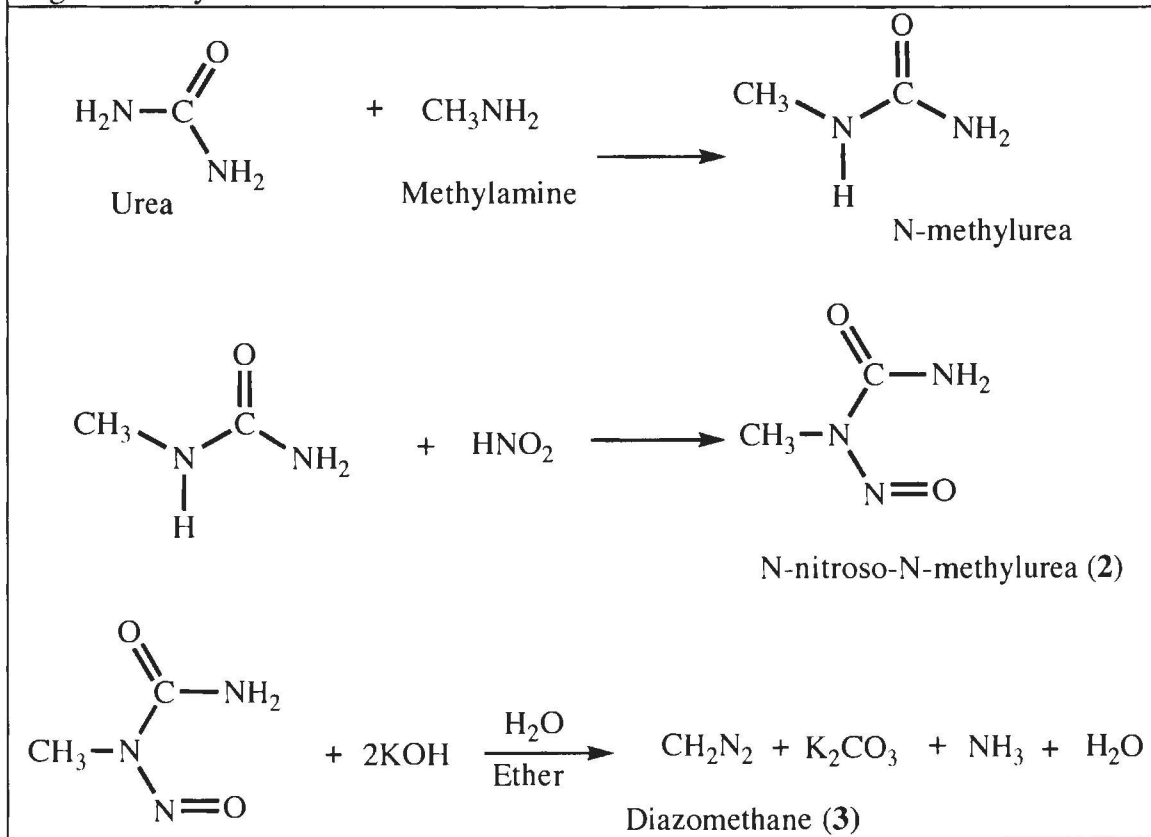
N-Nitrosomethylurea was prepared using a procedure described by Roue³⁴. Concentrated hydrochloric acid was added to methylamine solution (20 g of 24%) until the solution was acidic to pH paper (pH of 4-5) (the solution was also checked using methyl red indicator). Approximately 15 mL of HCl were required. Water was added to bring the total weight to 50 g. Urea (30 g) was added and the solution was refluxed for 3 h. The solution was cooled to room temperature and sodium nitrite (11 g) was added and the solution mixture was cooled to 0 °C. A mixture of ice (60 g) and concentrated sulfuric acid (10 g) in a 3 L beaker was cooled in an ice-salt bath. The methylurea-sodium nitrite solution was cooled in an ice bath and then slowly added to the cold sulfuric acid at such a rate that the temperature did not rise above 0 °C. The nitrosomethyl urea rose to the surface as a foamy precipitate which was filtered with suction filtration. Crystals were dried overnight to give 12.2 g (50% yield) of a light yellow solid.

The next step was to take the N-nitrosomethyl urea to synthesize the diazomethane (3).

Synthesis of diazomethane (3) (Figure A2)

Diazomethane was prepared using a procedure described by Roue³⁴. A 40% potassium hydroxide (3 mL) solution was added to ether (10 mL) and cooled to 5 °C. With continuous cooling and shaking, nitrosomethylurea (1.0 g) was added in small portions over a period of 1-2 minutes. The deep yellow ether layer was separated from the water layer and the ether layer containing diazomethane was used immediately for the methylation of 1-ethanoyl-3-methyl-2-thioxoimidazolidin-4-one (4); 0.30 g, (35%) of the diazomethane was recovered and used without further purification for the next step.

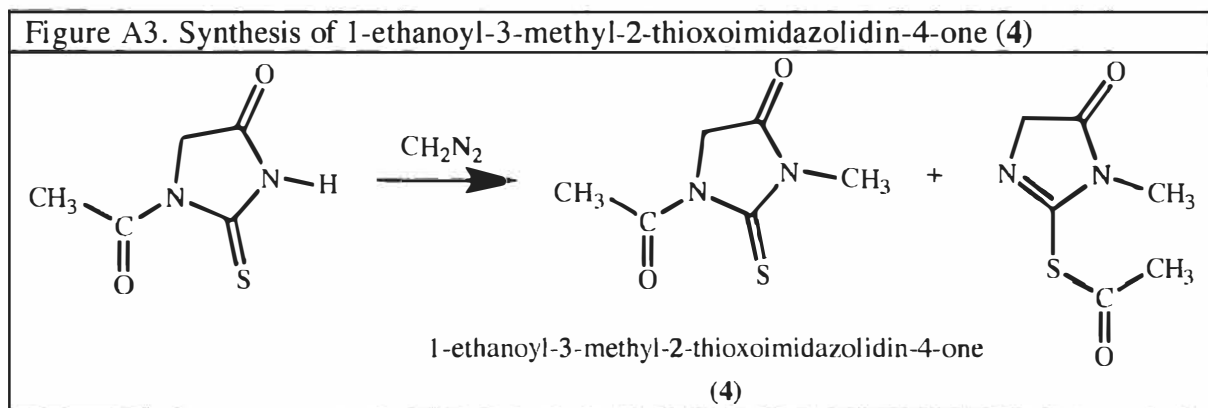
Figure A2. Synthesis of diazomethane.



The next step to this synthesis was to react compound (1) from figure 30 with the diazomethane from Figure A1 to end up with (4) (Figure A2) after purification of the same.

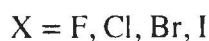
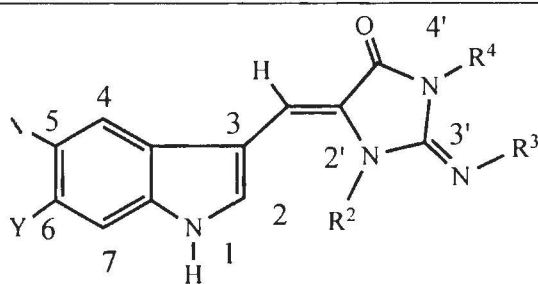
Synthesis of 1-ethanoyl-3-methyl-2-thioxoimidazolidin-4-one (4) (Figure A3)

The methylation of acetylthiohydantoin was carried out using a procedure described by Arenal ²². Acetylthiohydantoin was added to an ether solution of diazomethane (10 mL, 0.40 g). The reaction was kept at room temperature for 5 minutes and the ether was evaporated to give a light brown solid; 0.30 g of this brown solid was recovered. Thin layer chromatography (TLC) using a 50:50 (benzene:ethyl acetate) solution as an eluent showed that the solid was a mixture of two compounds. The mixture was separated by column chromatography using benzene and ethylacetate (17:3) as the eluent. A light yellow powder was obtained; 0.23 g of this yellow powder was recovered and it had a m.p. 145-146 °C.

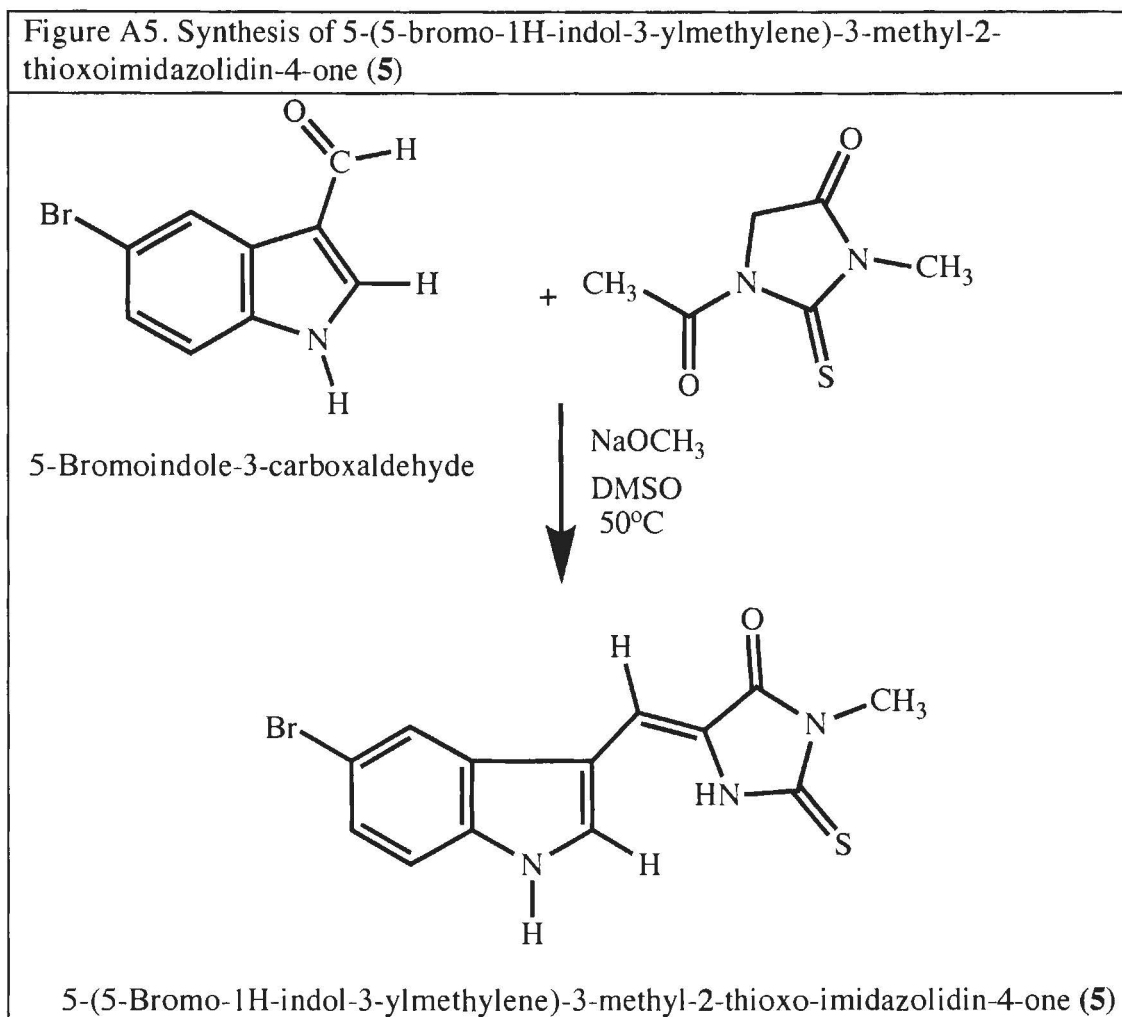


Once compound (4) was purified and isolated, it was then used for the following step, the condensation of the indole ring with the imidazolidine. At this point of the research, the goal was to come out with a synthetic procedure that could be used to synthesize all of the aplysinopsin analogs. It is also important to note that, at this time, we were looking for a general procedure that could work across the board with minor modification. For this reason, it was decided to use an indole-3-carboxaldehyde that was commercially available before embarking on the synthesis of the more complex indole aldehydes required for this project. It was for this reason that the 5-bromoindole-3-carboxaldehyde and the 5-chloroindole-3-carboxaldehyde were used, both of which are commercially available. It is also important to mention that the target molecule has a halogen (Br, Cl, F, I) (see Figure A4) in the six position instead of the five position, but once again the indoles used at this point have the halogen in the five position because they are commercially available eliminating several synthetic steps at this stage in our research.

Figure A4. Numbering system of aplysinopsin

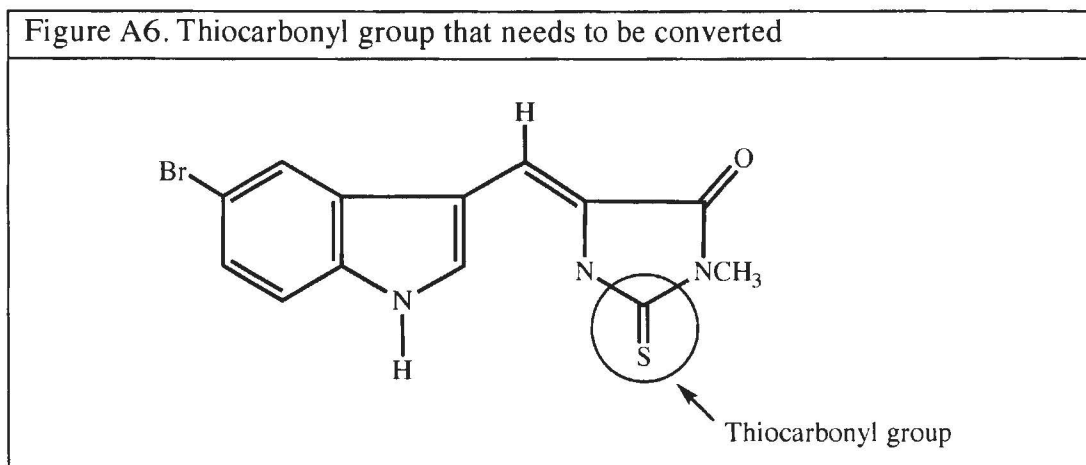


Condensation of 5-bromoindole-3-carboxaldehyde with (4) (Figure A5) gave 5-((5-bromo-1H-indol-3-yl)methylene)-3-methyl-2-thioxo-imidazolidin-4-one (5).



The next step in the synthetic scheme was the conversion of a thiocarbonyl group (Figure A6) in a 3-methylimidazolidine-2,4-dione, a reaction that has been reported using a similar starting material³⁵. It was necessary to convert the thiocarbonyl group to an

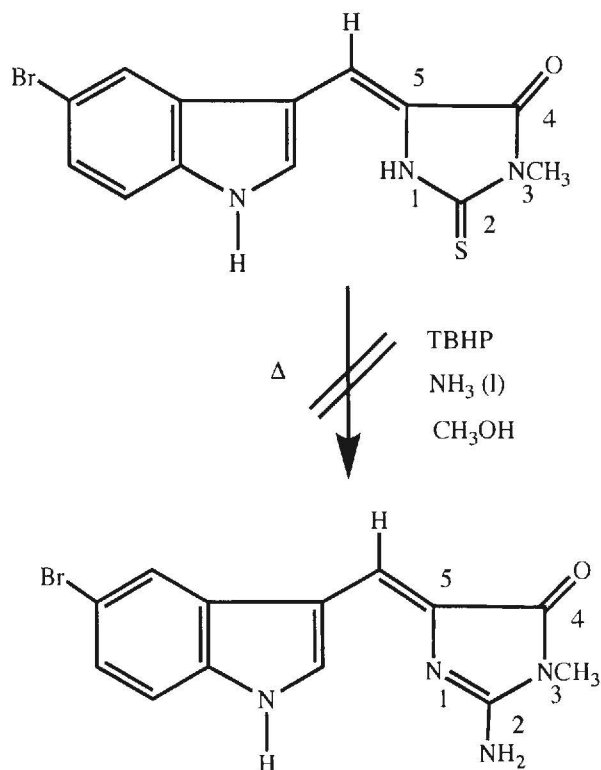
imino (NH) group, which is known to be required for binding to 5-HT receptors 2A and 2C Hu *et al*²¹.



The conversion of a thiocarbonyl group in a 3-methylimidazolidine-2,4-dione has been reported³³. However, when the same procedure was used in our product, this conversion did not take place. Instead, this procedure gave a black tarry semisolid indicating that the indole ring had undergone oxidation (Figure A7).

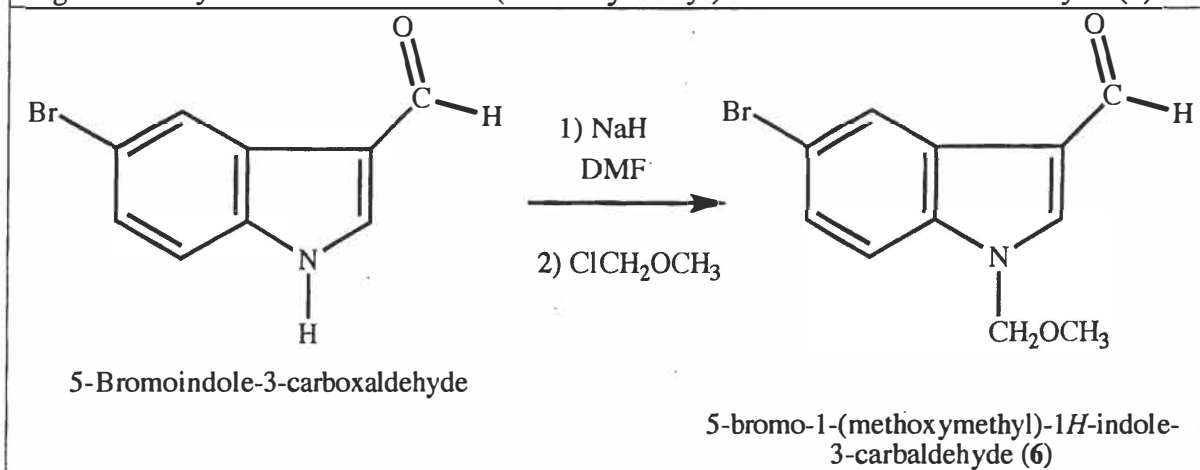
There is some indication³⁵ that a protecting group on the indole nitrogen inhibits oxidation. Therefore, the 5-bromoindole-3-carboxaldehyde was converted into 5-bromo-1-(methoxymethyl)-1H-indole-3-carboxaldehyde (**6**) by reaction with chloromethyl methylether. 5-bromo-1-(methoxymethyl)-1H-indole-3-carboxaldehyde failed to undergo condensation with creatinine (Figure A8).

Figure A7. Attempted synthesis of 2-amino-5-((5-bromo-1H-indol-3-yl)methylene)-3-methyl-3,5-dihydro-imidazol-4-one.



2-Amino-5-((5-bromo-1H-indol-3-yl)methylene)-3-methyl-3,5-dihydro-imidazol-4-one

Figure A8. Synthesis of 5-bromo-1-(methoxymethyl)-1H-indole-3-carboxaldehyde (6).



Synthesis of 5-bromo-1-(methoxymethyl)-1H-indole-3-carboxaldehyde (6)

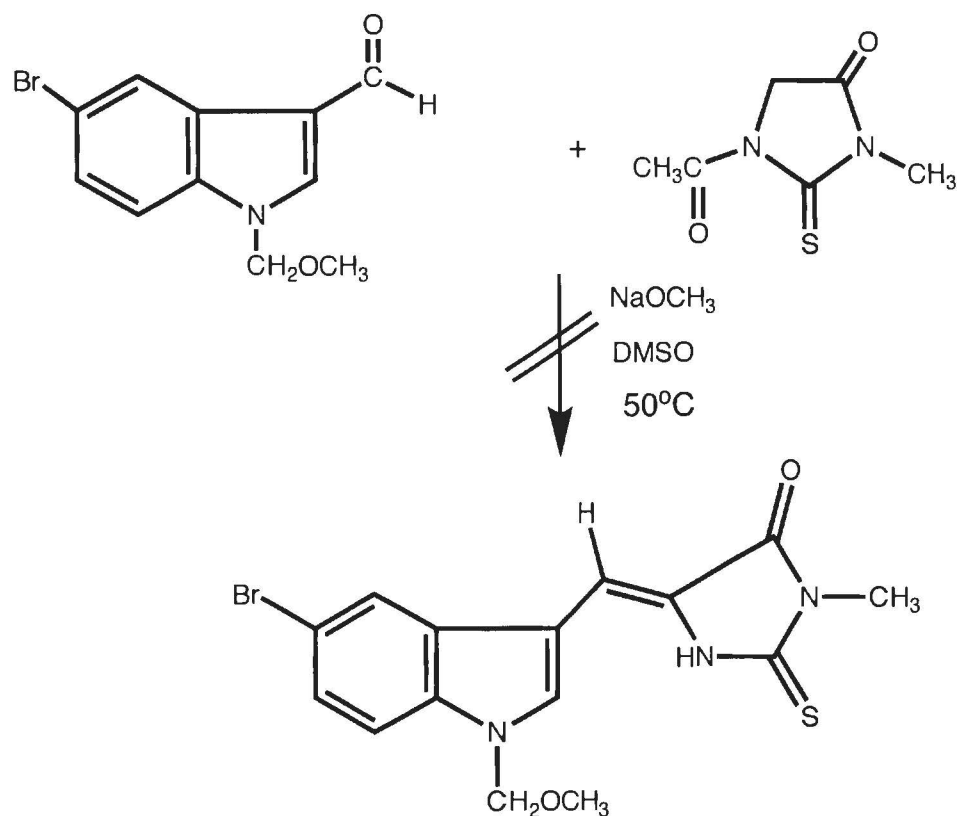
A suspension of NaH (0.53 g) in dry (dimethyl formamide) DMF (30 mL) was added dropwise to a solution of 5-bromo indole-3-carboxaldehyde (5 g) in DMF (10 mL). The solution mixture was stirred at room temperature for 1 h. The solution was cooled to 0 °C and chloromethyl methyl ether (1.80 g) was slowly added. The solution was allowed to cool at room temperature and stirred for an additional 1 h. The solution was poured onto iced-water and the precipitate was collected by filtration, air-dried and recrystallized from ethylacetate/hexane to give a cream powder; 3.5 g of this powder were collected and it had a m.p. 82.5-83.8 °C.

Synthesis of (Z)-5-((5-bromo-1-(methoxymethyl)-1H-indol-3-yl)methylene)-3-methyl-2-thioxoimidazolidin-4-one

5-Bromoindole-1-(methoxymethyl)-1H-indole-3-carboxaldehyde (1.55 g) was added to a solution of 1-ethanoyl-3-methyl-2-thioxoimidazolidin-4-one (0.211 g) in

DMSO (2.0 mL) and sodium methoxide (0.028 g) in methanol (2.0 mL). The solution was heated to 50 °C for 1h. The solution was then cooled to room temperature and added to iced-water. The solution was extracted with ether and sodium chloride; the organic layer was dried over magnesium sulfate and the ether was removed by rotary evaporation. This reaction did not work and only starting material was recovered (Figure A9).

Figure A9. Attempted synthesis of (Z)-5-((5-bromo-1-(methoxymethyl)-1H-indol-3-yl)methylene)-3-methyl-2-thioxoimidazolidin-4-one

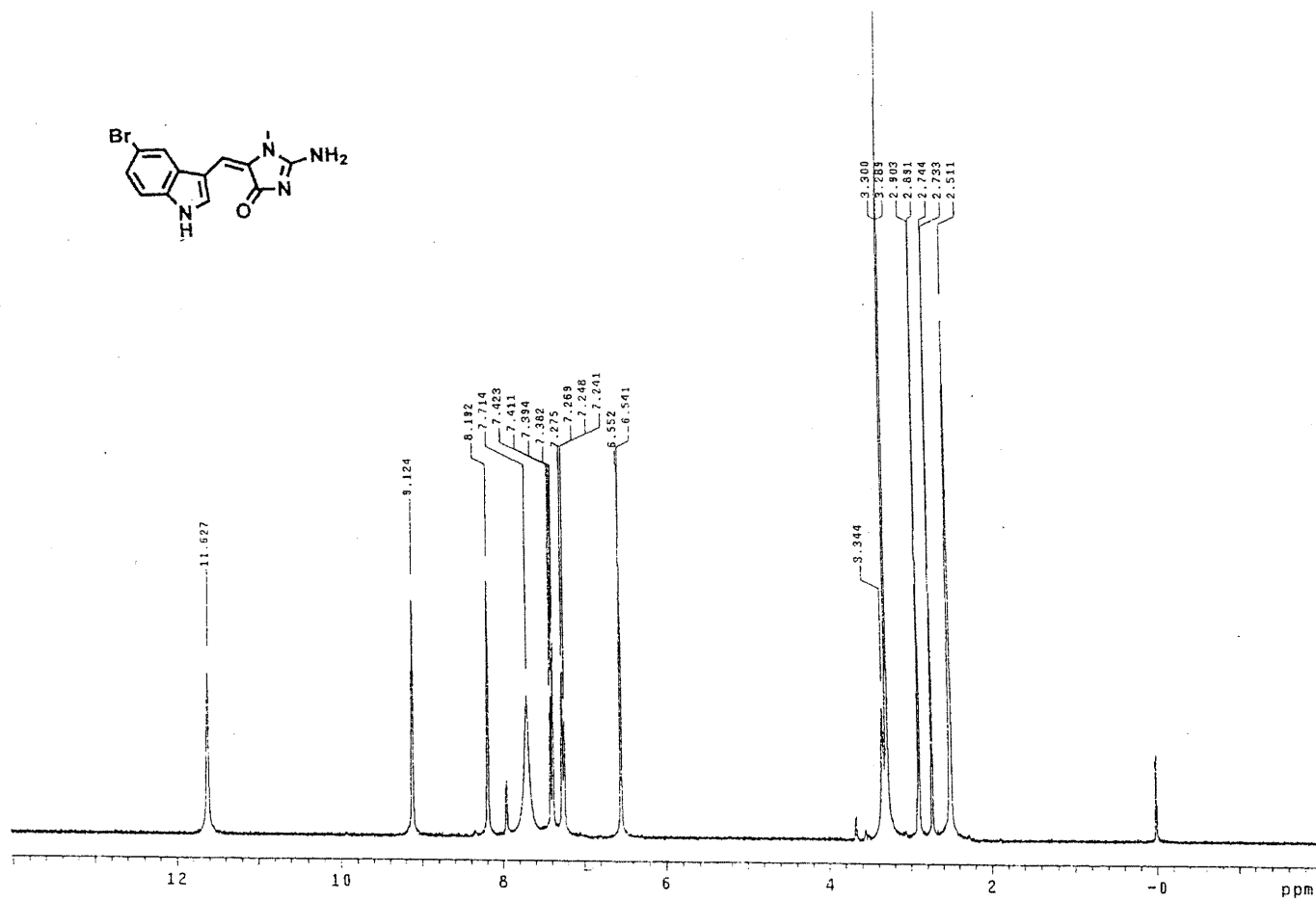
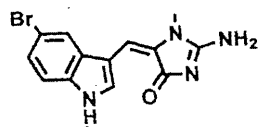


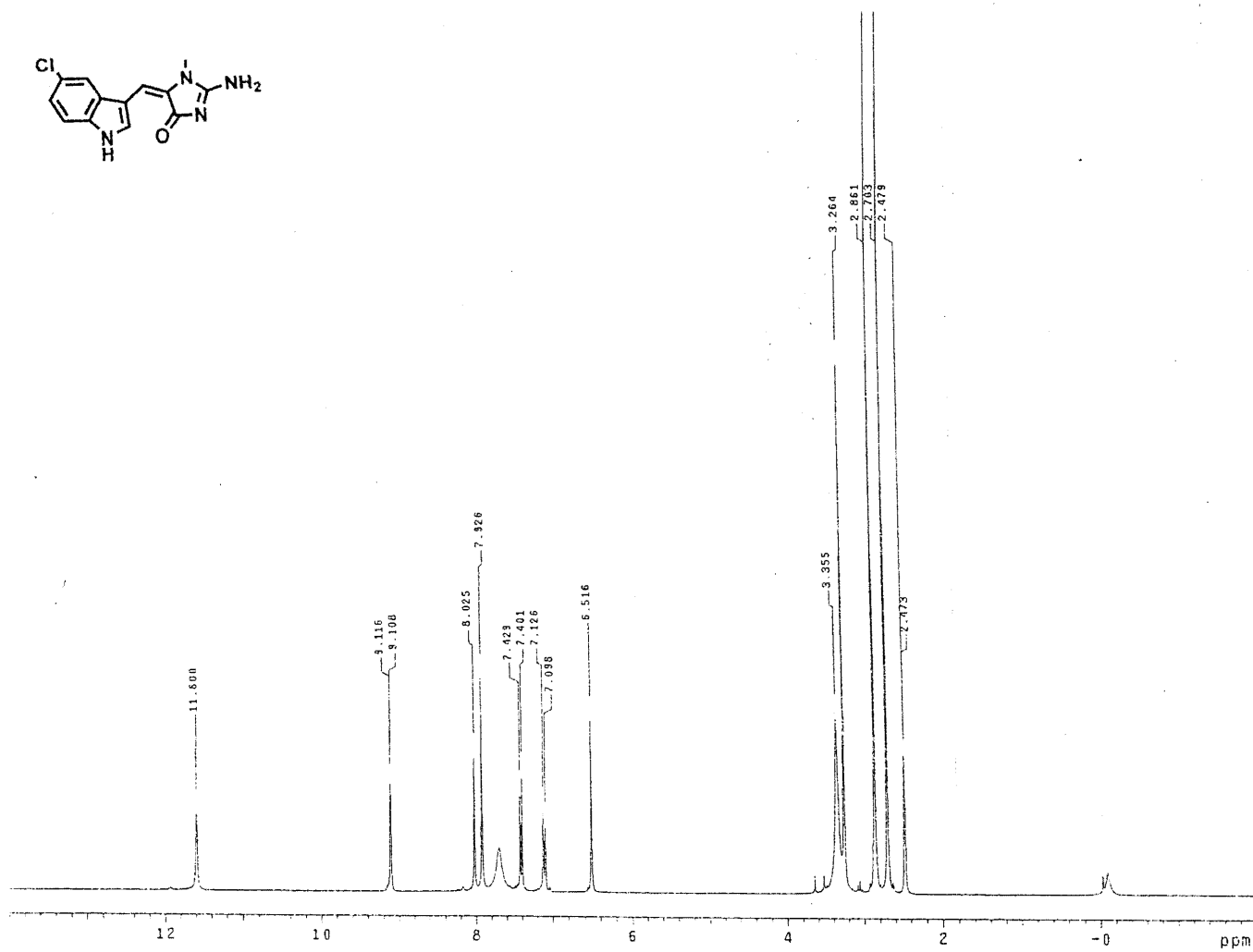
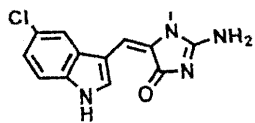
(Z)-5-((5-bromo-1-(methoxymethyl)-1H-indol-3-yl)methylene)-3-methyl-2-thioxoimidazolidin-4-one

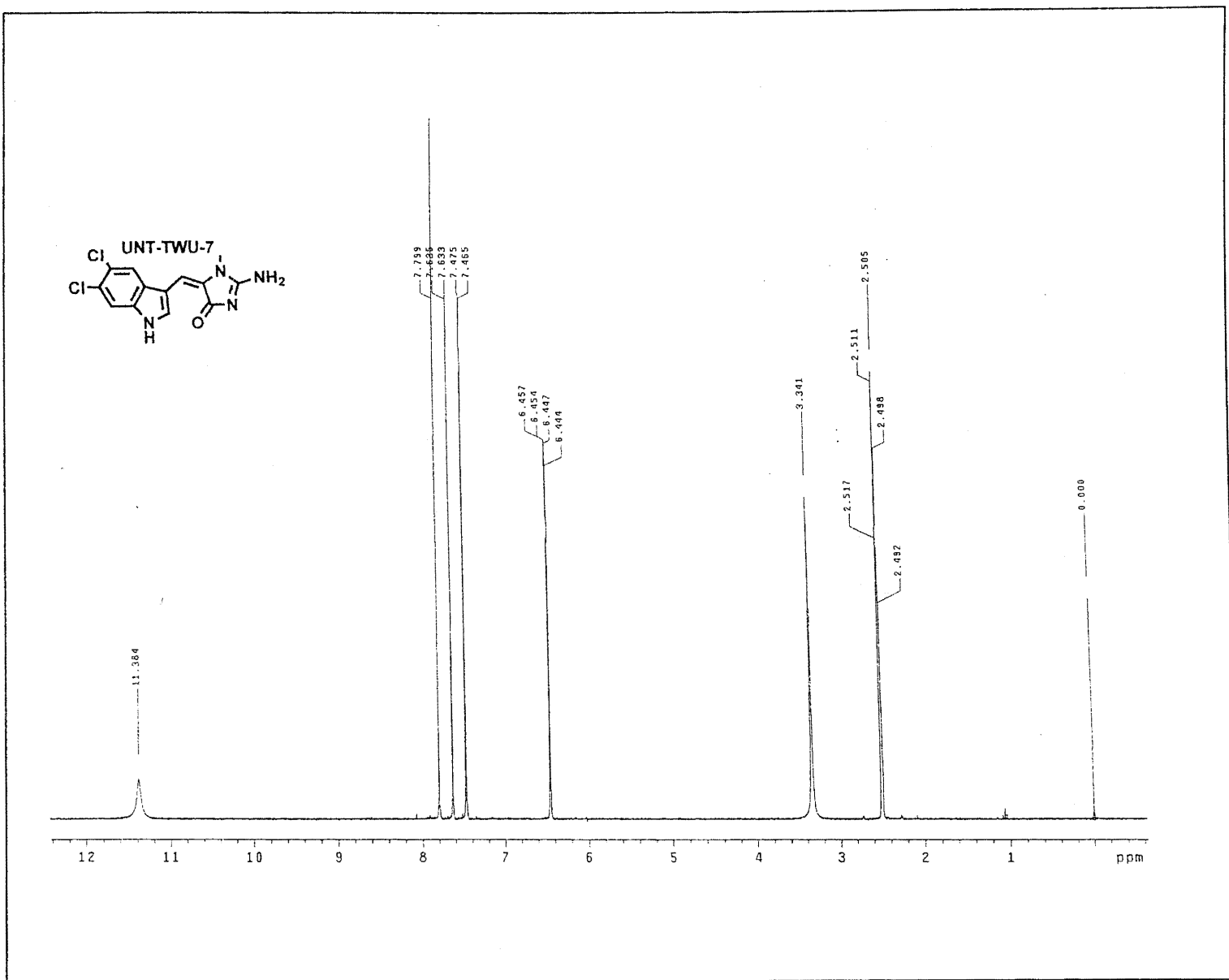
Another attempt to synthesize aplysinopsin analogs involved the preparation of 5-bromo or 5-chloro (1H-indol-3yl)methylene)-3-methylimidazolidine-2,4-dione by the condensation of 3-methyl imidazolidin-2,4-dione (**7**) with 5-chloro and 5-bromoindole-3-carboxaldehyde. Attempts to react 5-bromo or 5-chloro (1H-indol-3yl)methylene)-3-methylimidazolidine-2,4-dione with phosphorous pentachloride or phosphorous oxychloride followed by the addition of ammonia failed to give aplysinopsin analogs. Unreacted starting material was recovered in these reactions.

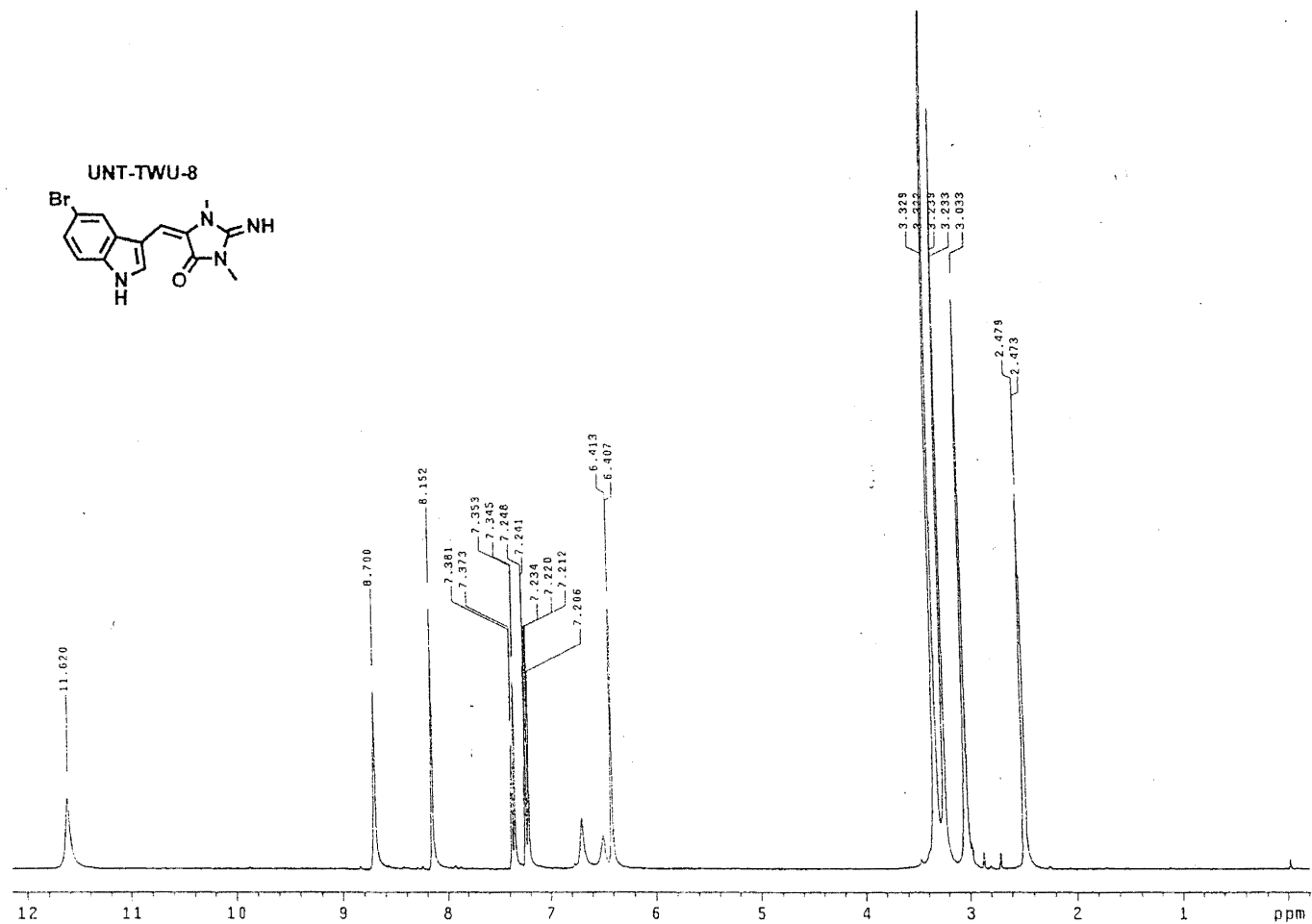
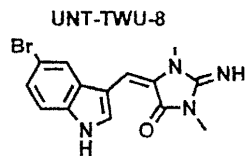
APPENDIX B

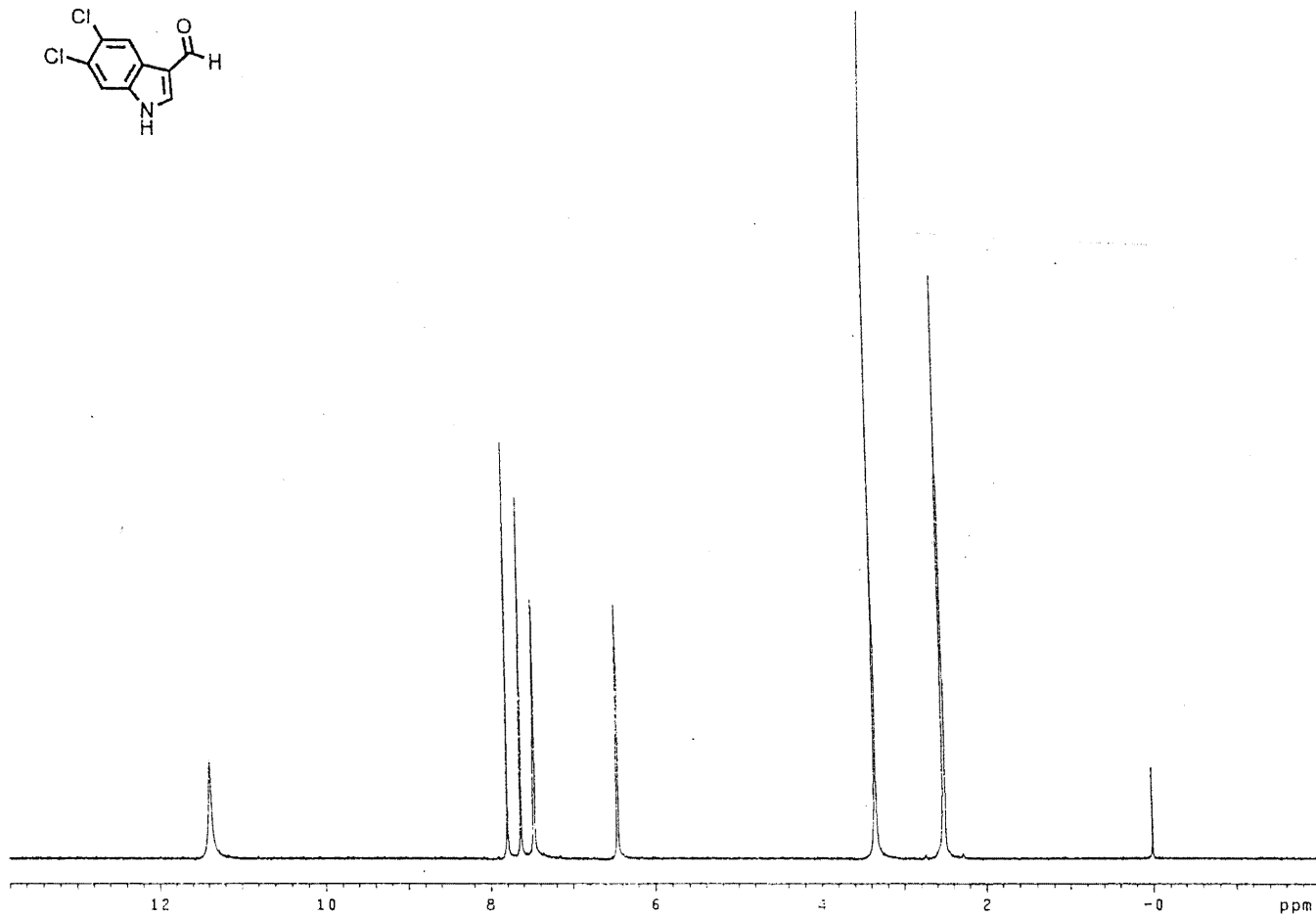
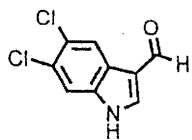
NMR Spectra

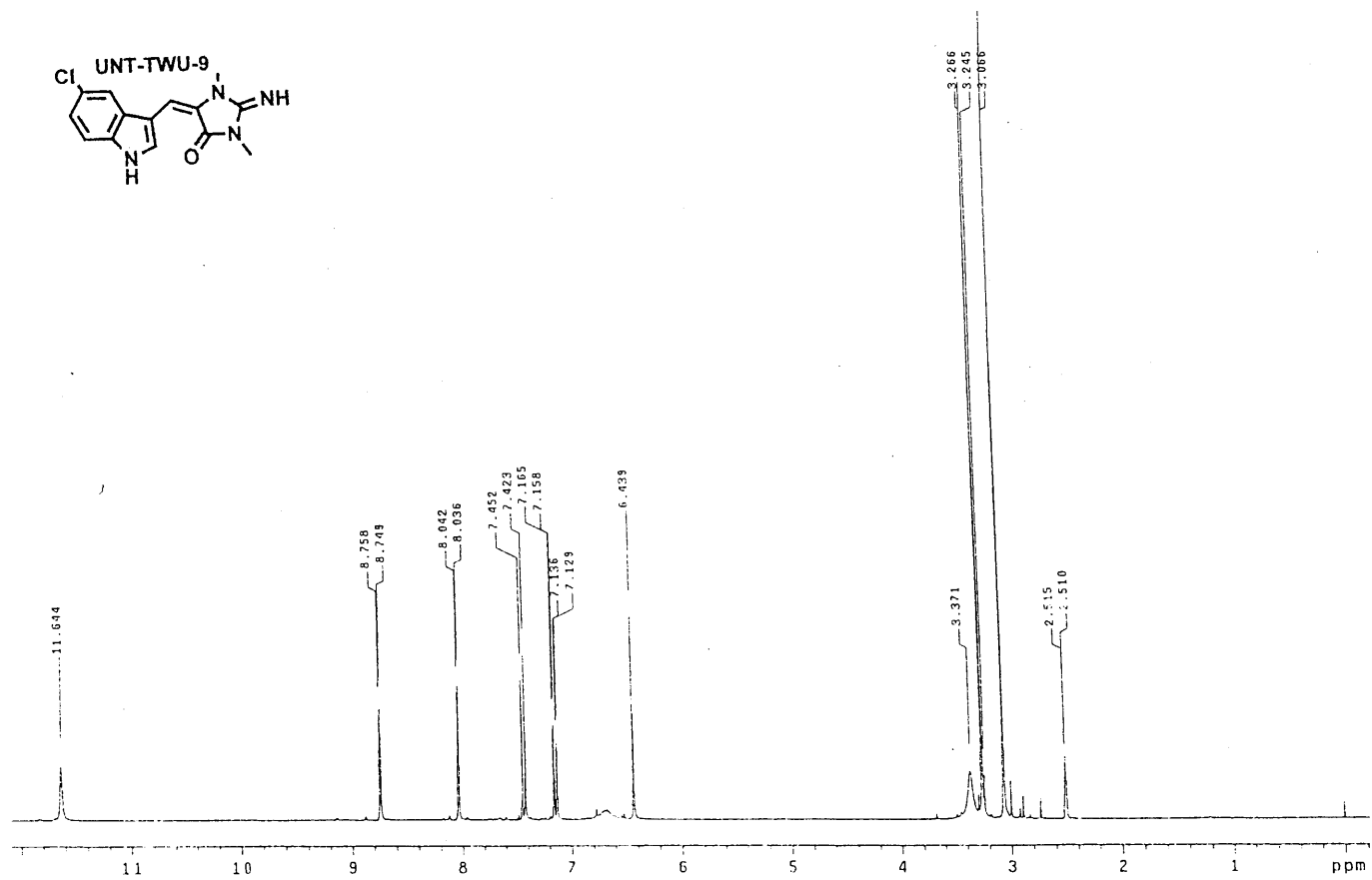


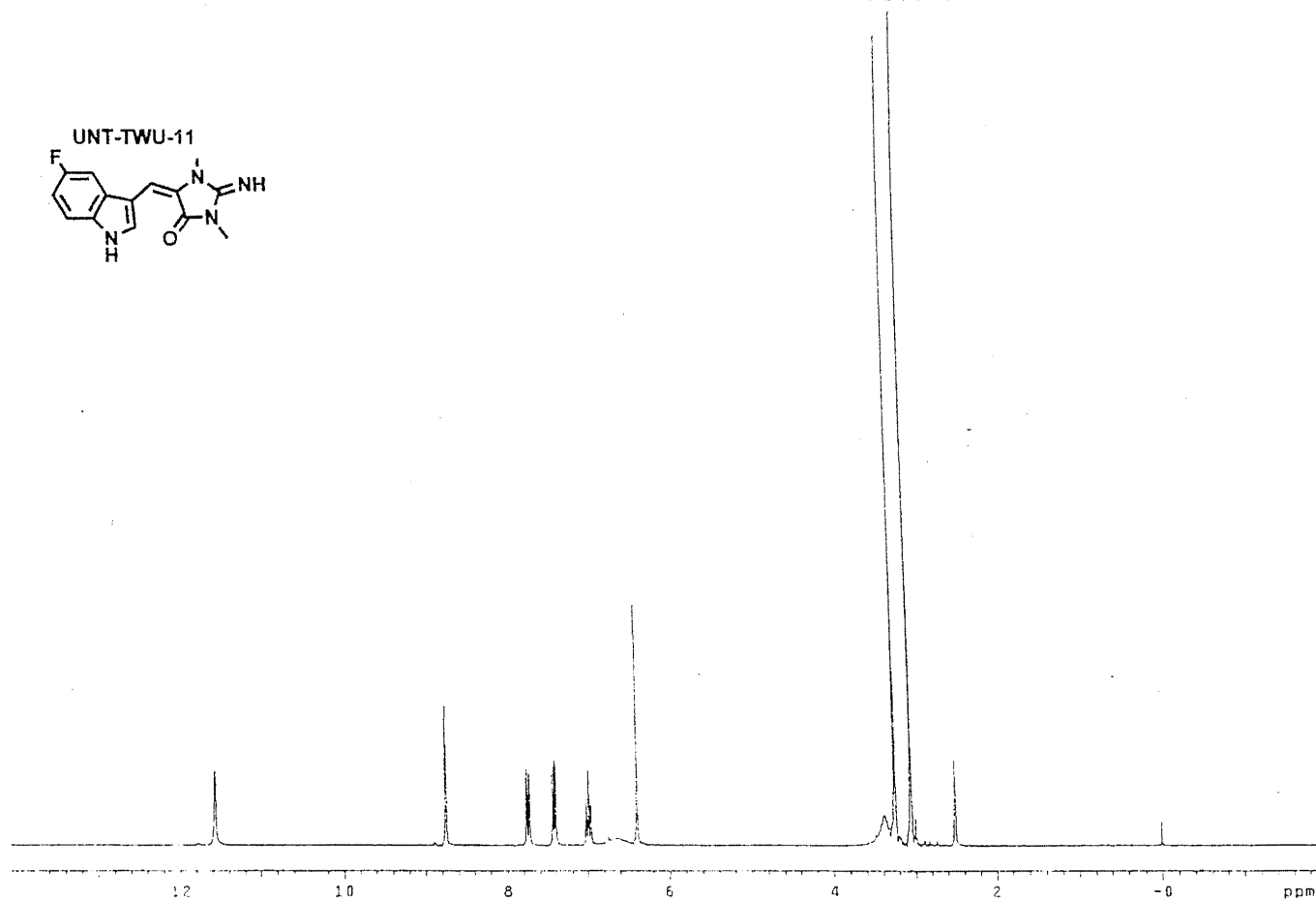
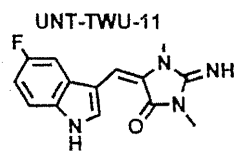


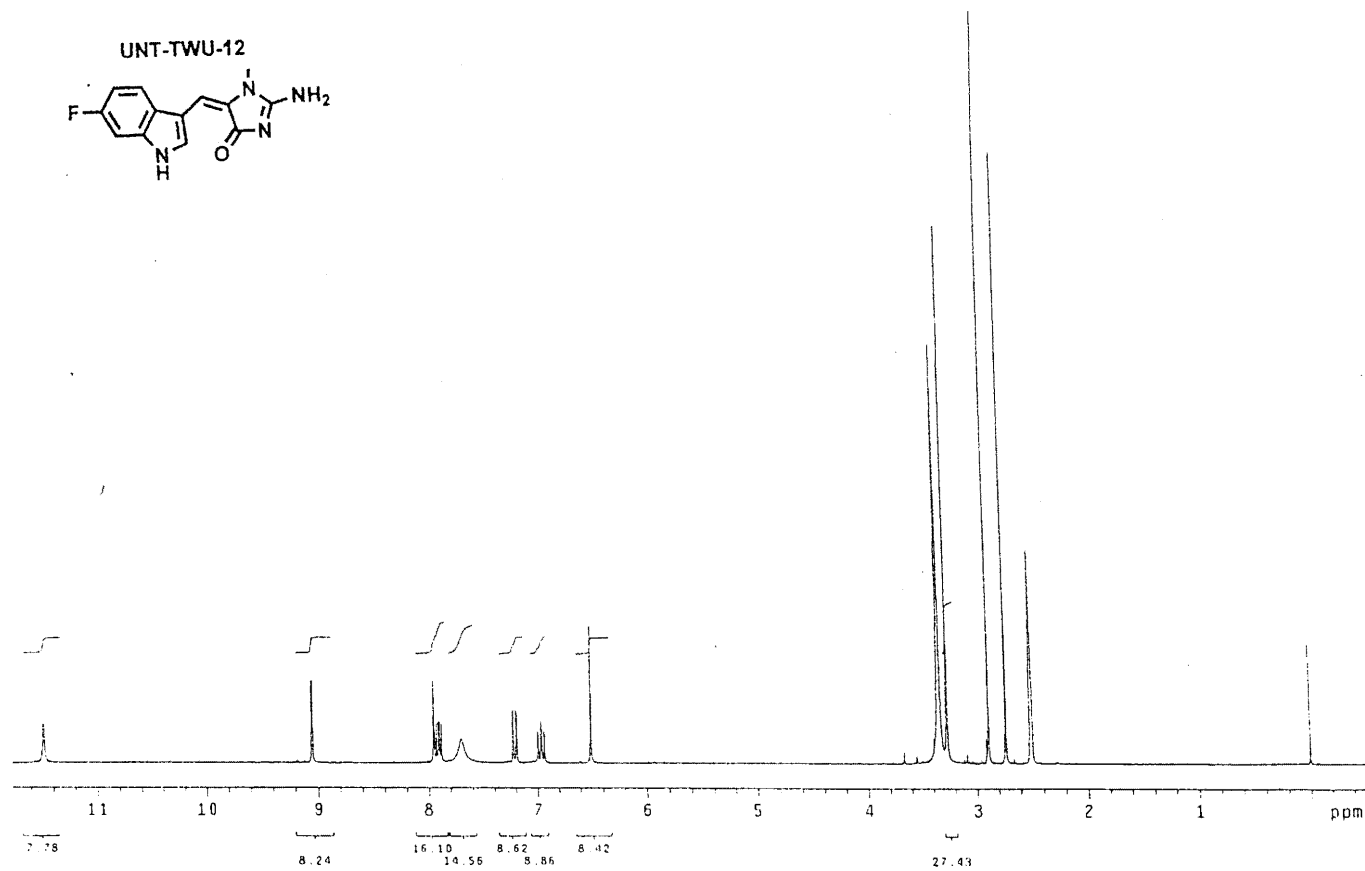






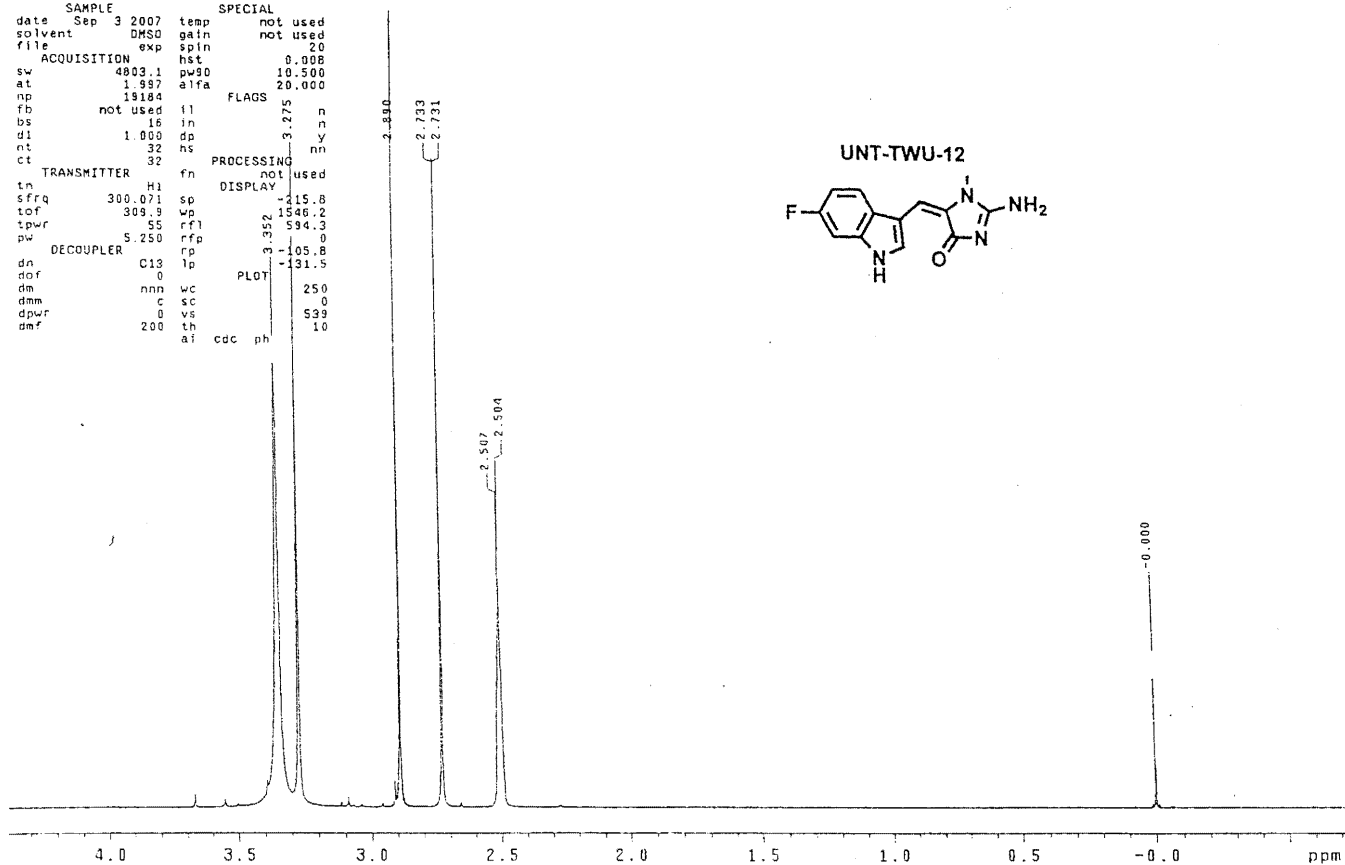
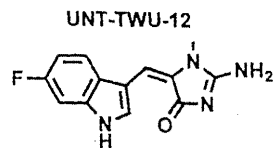


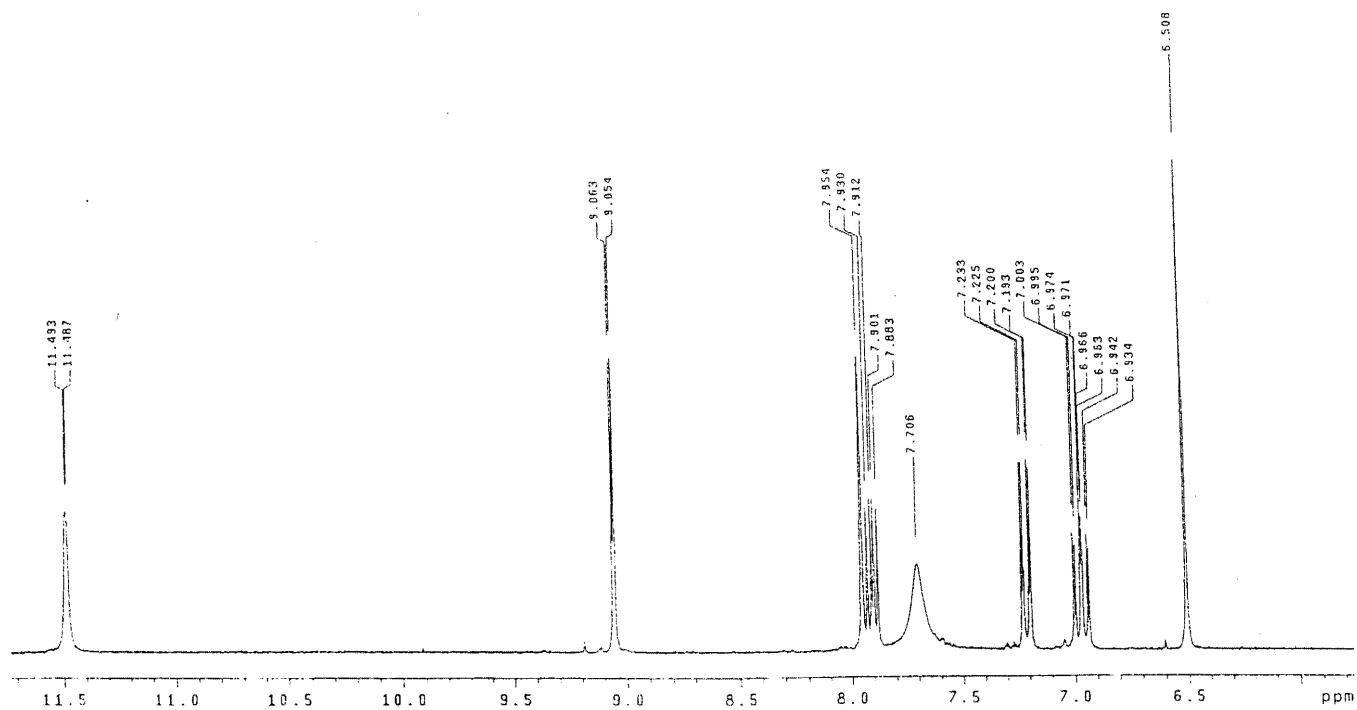
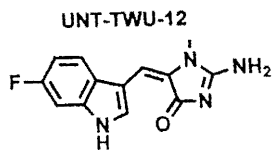


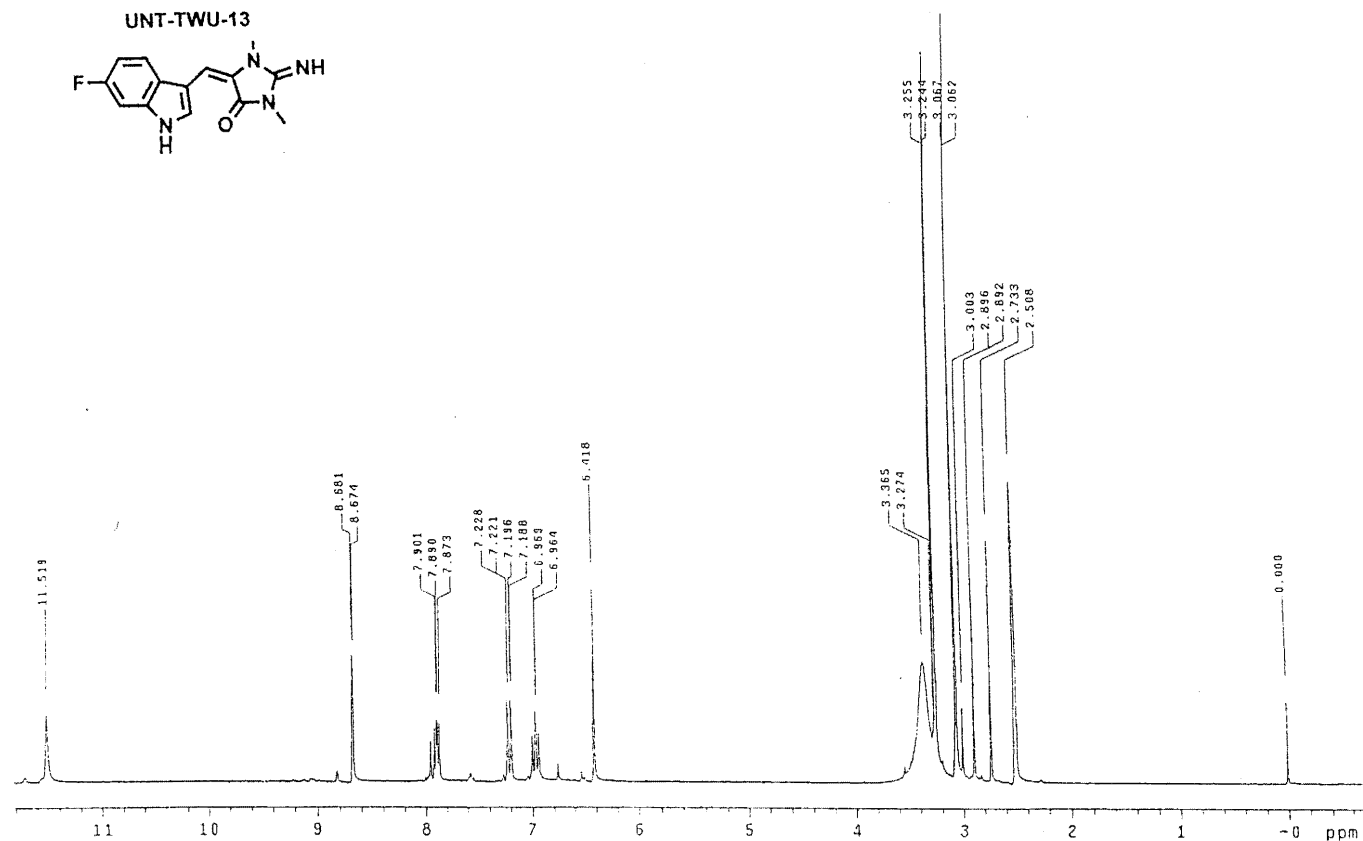


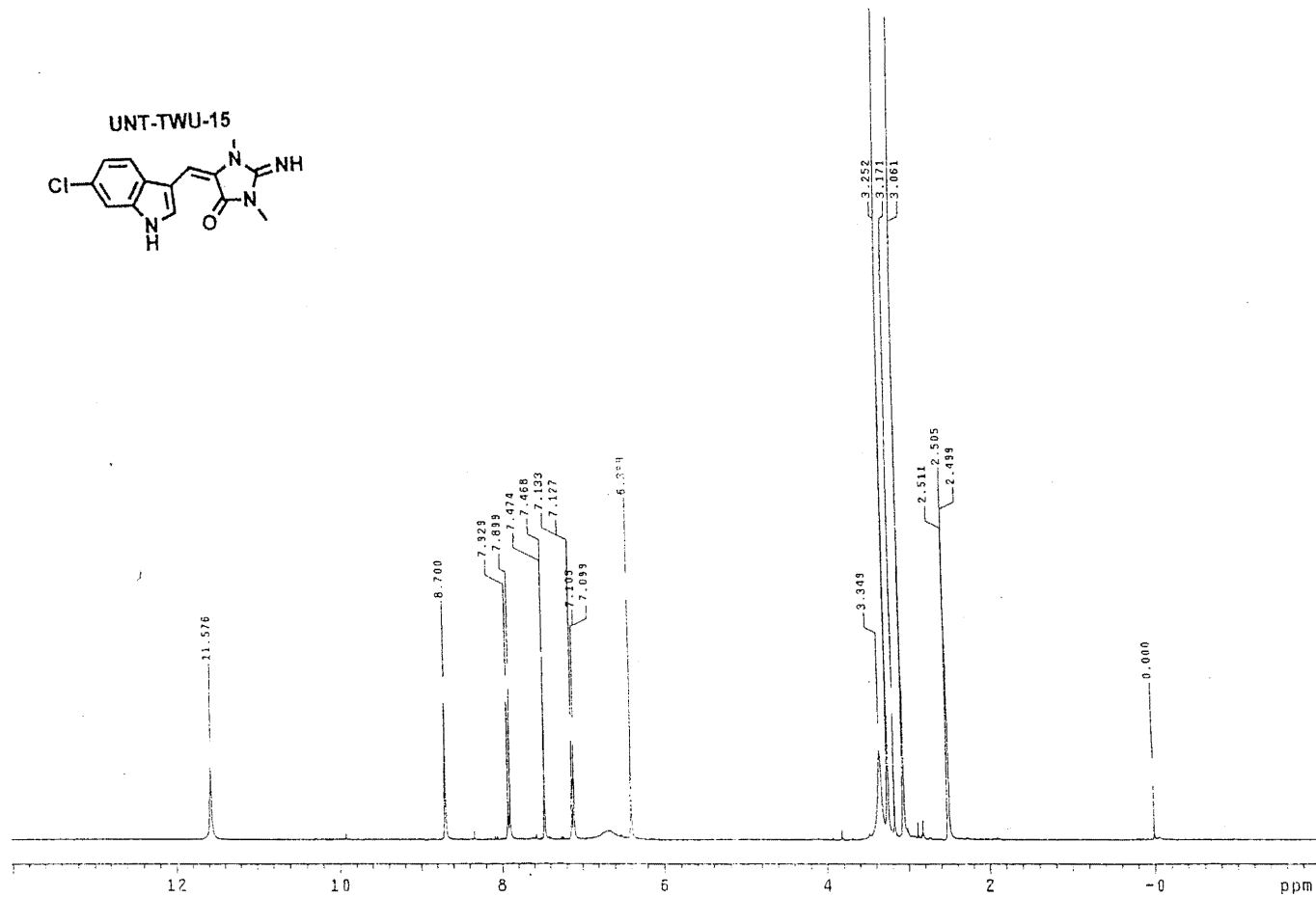
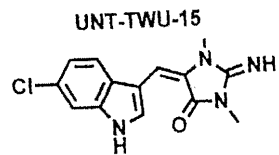
expl s2pu1

SAMPLE		SPECIAL	
date	Sep 3 2007	temp	not used
solvent	DMSO	gain	not used
file	exp	spin	20
ACQUISITION		hst	0.008
sw	4803.1	pw90	10.500
at	1.997	aifa	20.000
np	19184	FLAGS	
fb	not used	fl	n
bs	16	in	n
d1	1.000	dp	y
nt	32	hs	nn
ct	32	PROCESSING	
TRANSMITTER		fn	not used
tn	H1	DISPLAY	
sfrq	300.071	sp	215.8
tof	309.3	wp	1546.2
tpwr	55	rfl	594.3
pw	5.250	rfa	0
DECOUPLER		rp	105.8
dn	C13	lp	131.5
dof	0	PLOT	
dm	nnn	wc	250
dmm	c	sc	0
dpwr	0	vs	539
dmf	200	th	10
	ai	cdc	ph

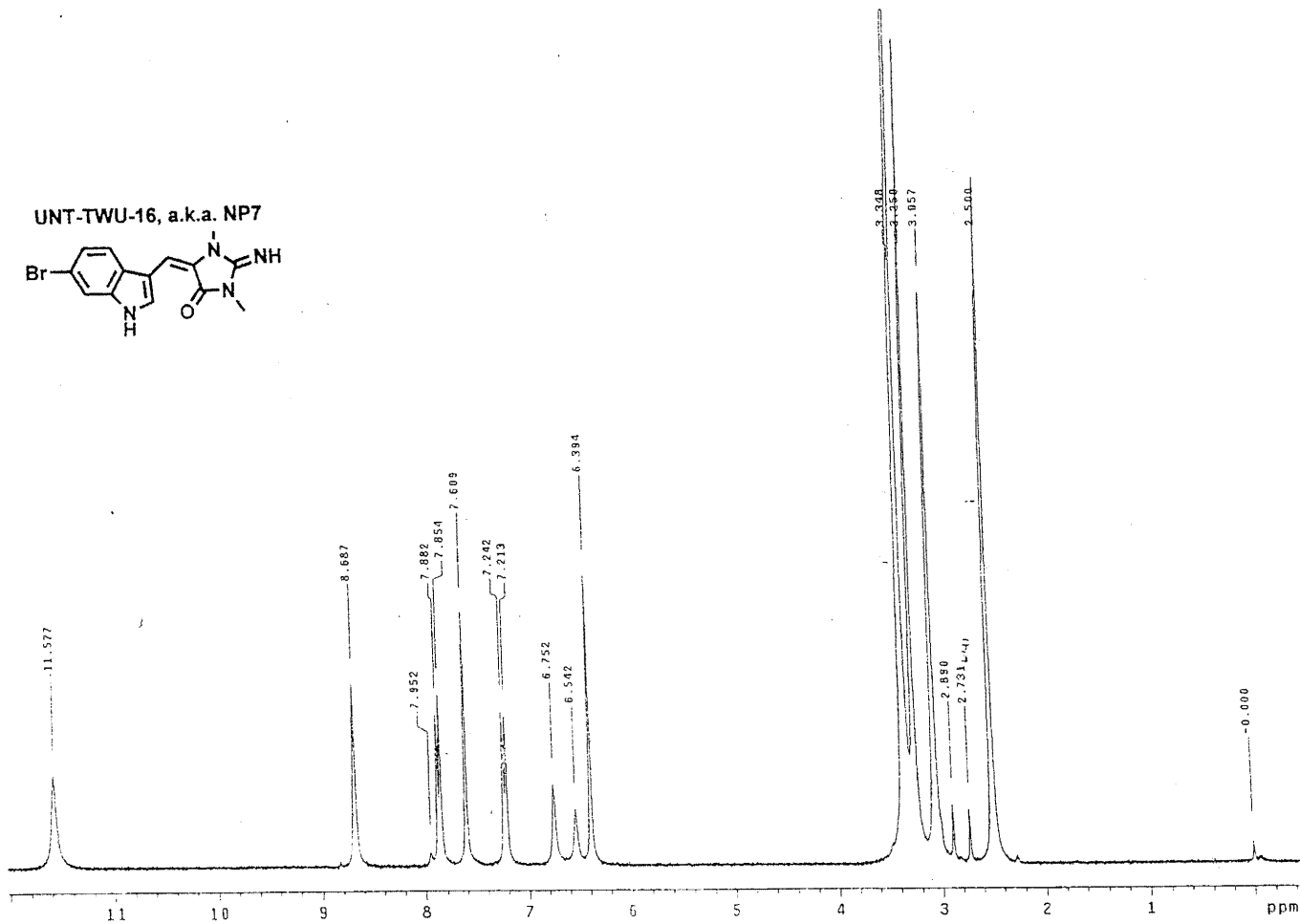
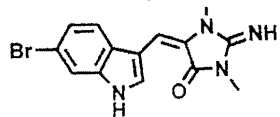


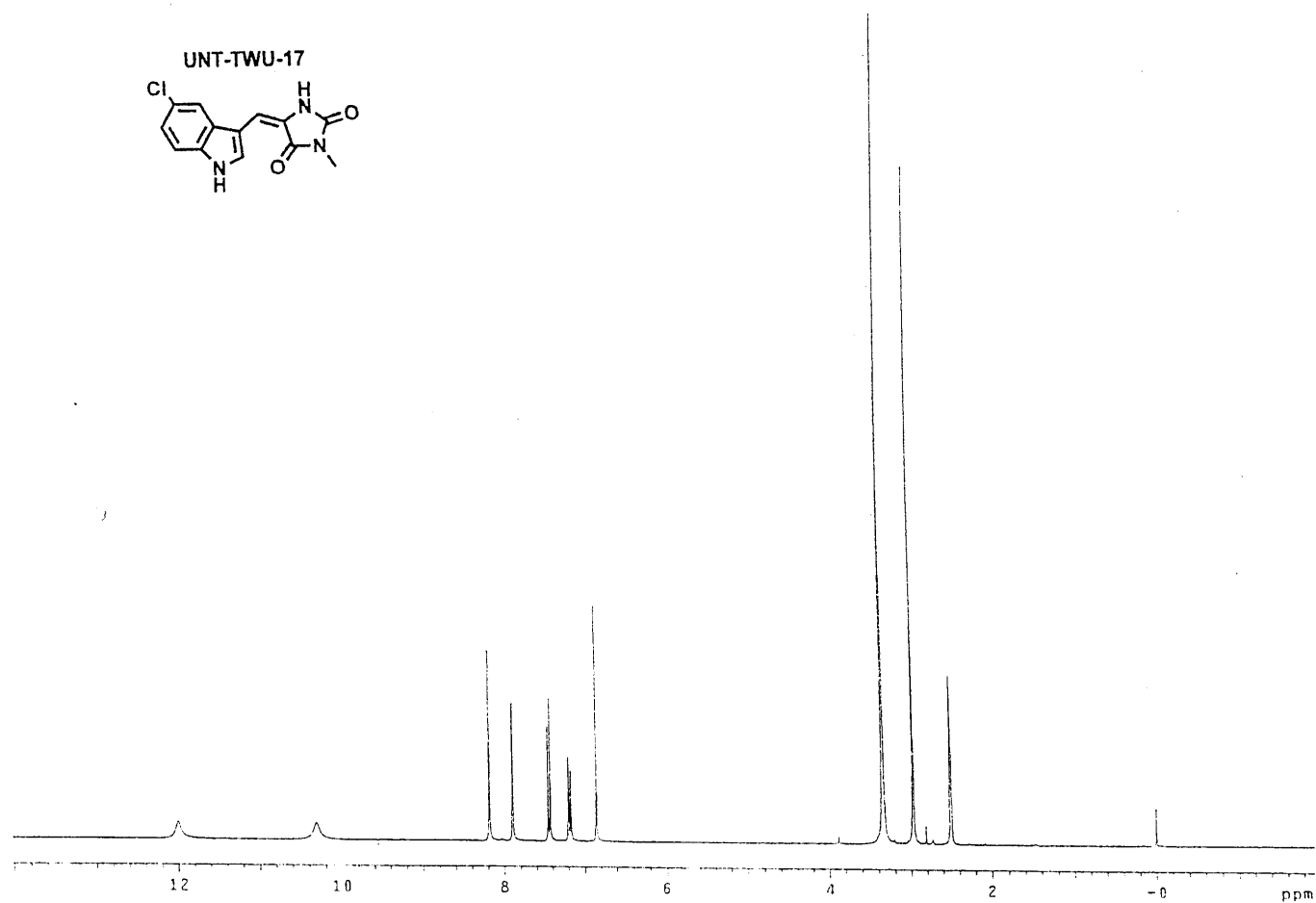
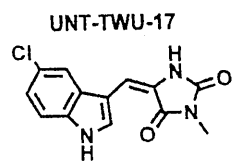


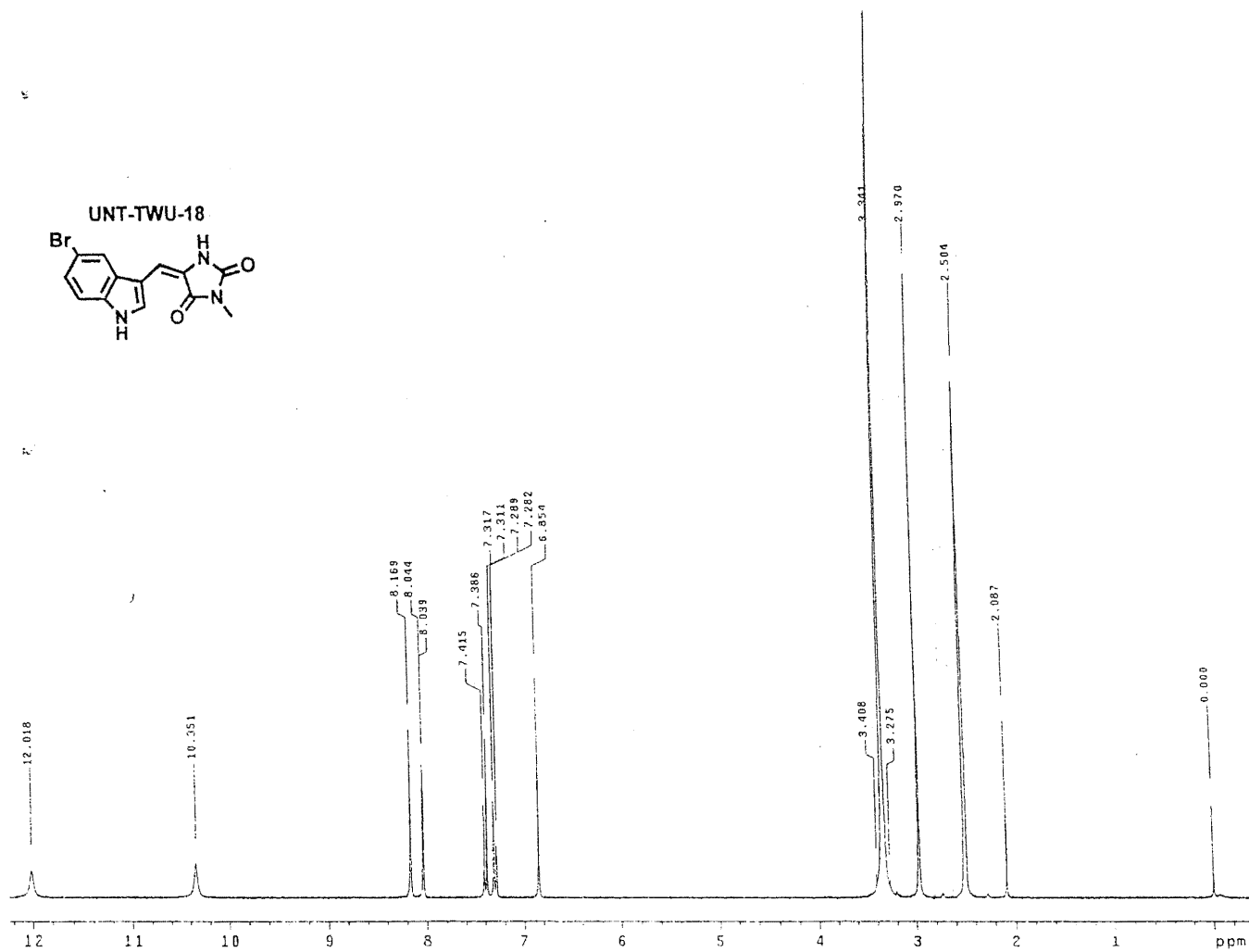
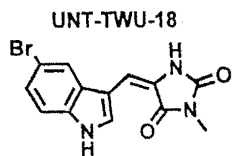


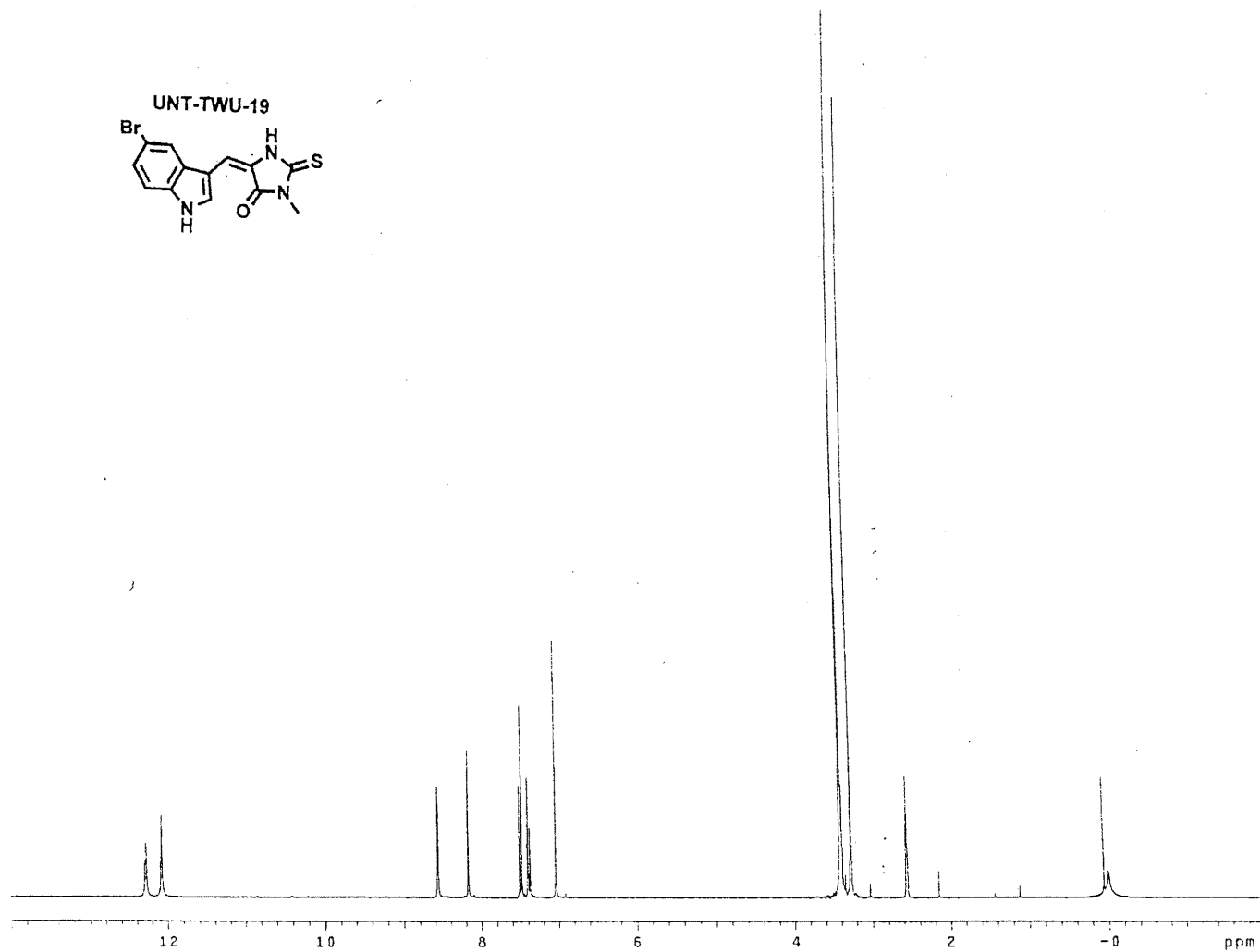
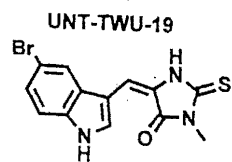


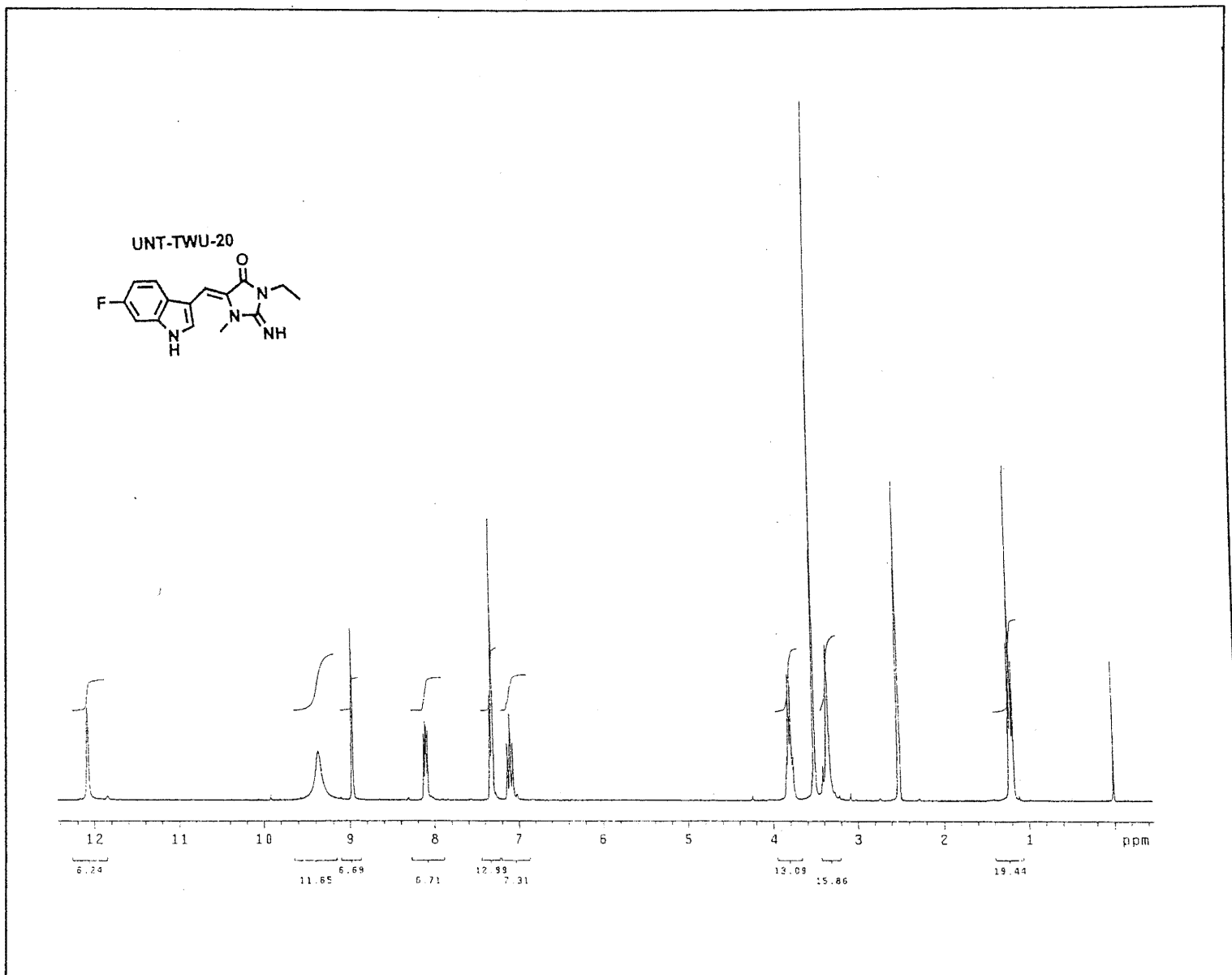
UNT-TWU-16, a.k.a. NP7

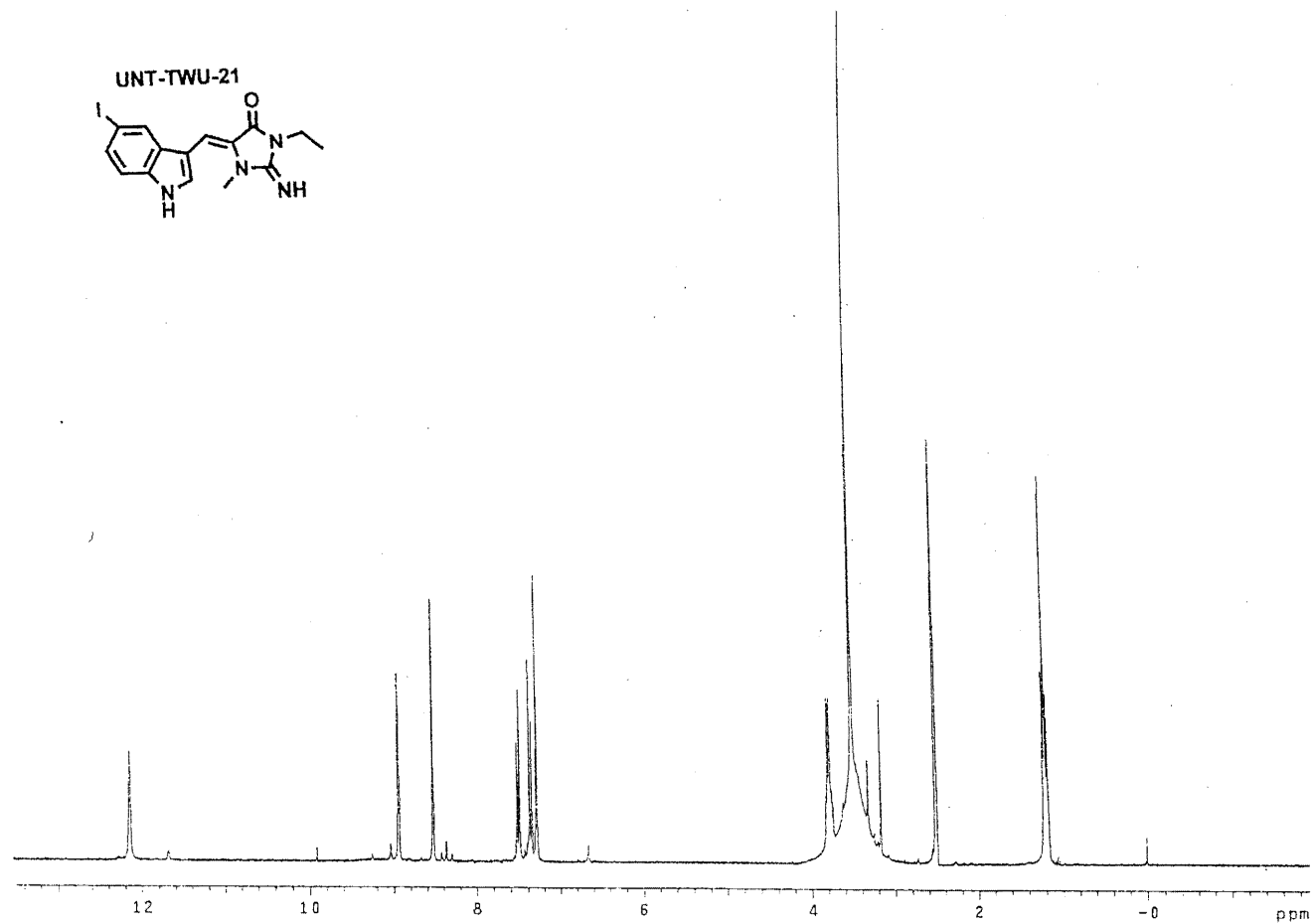
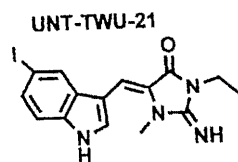


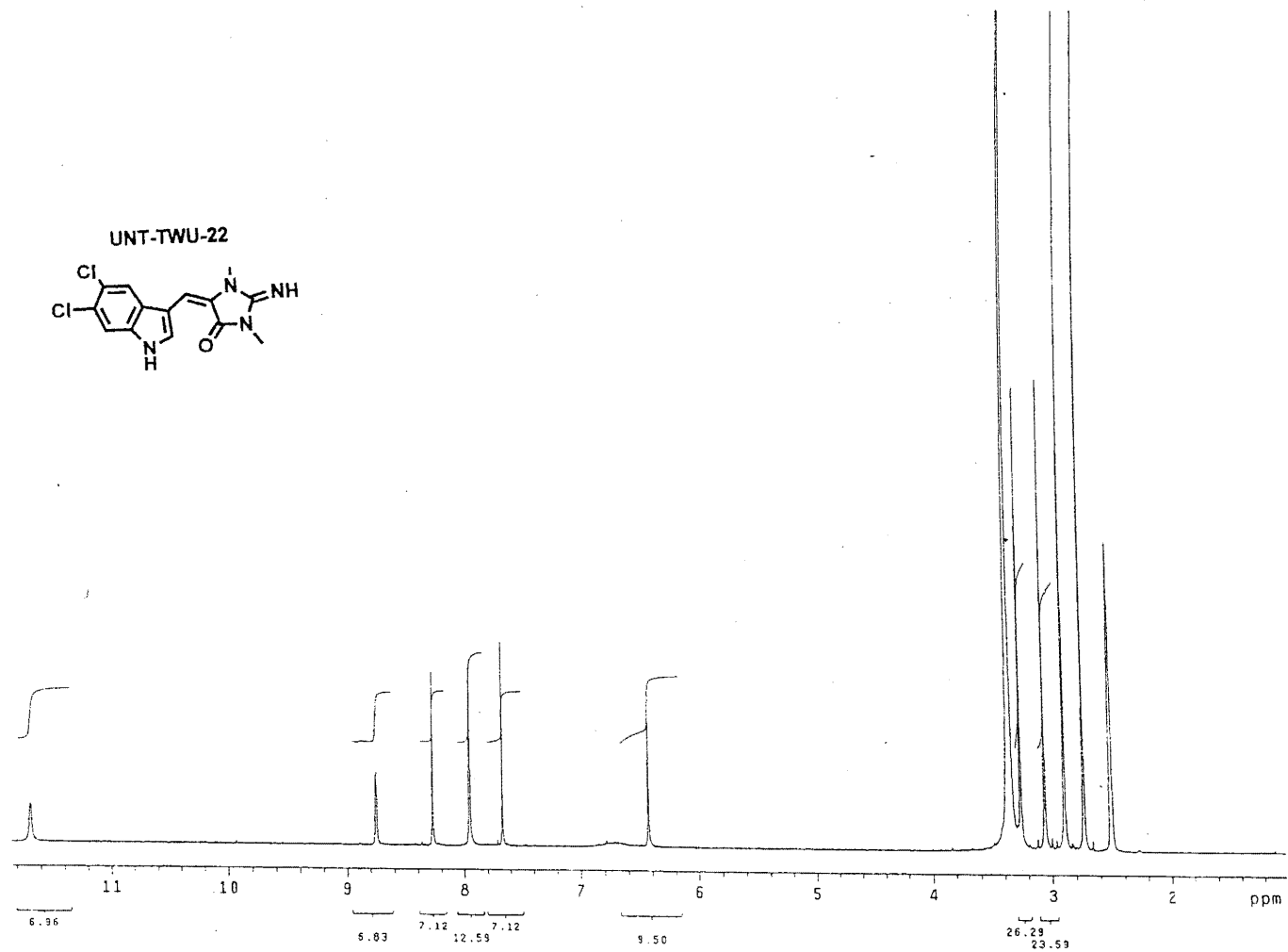
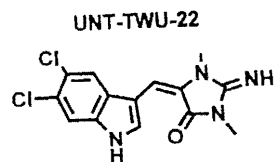


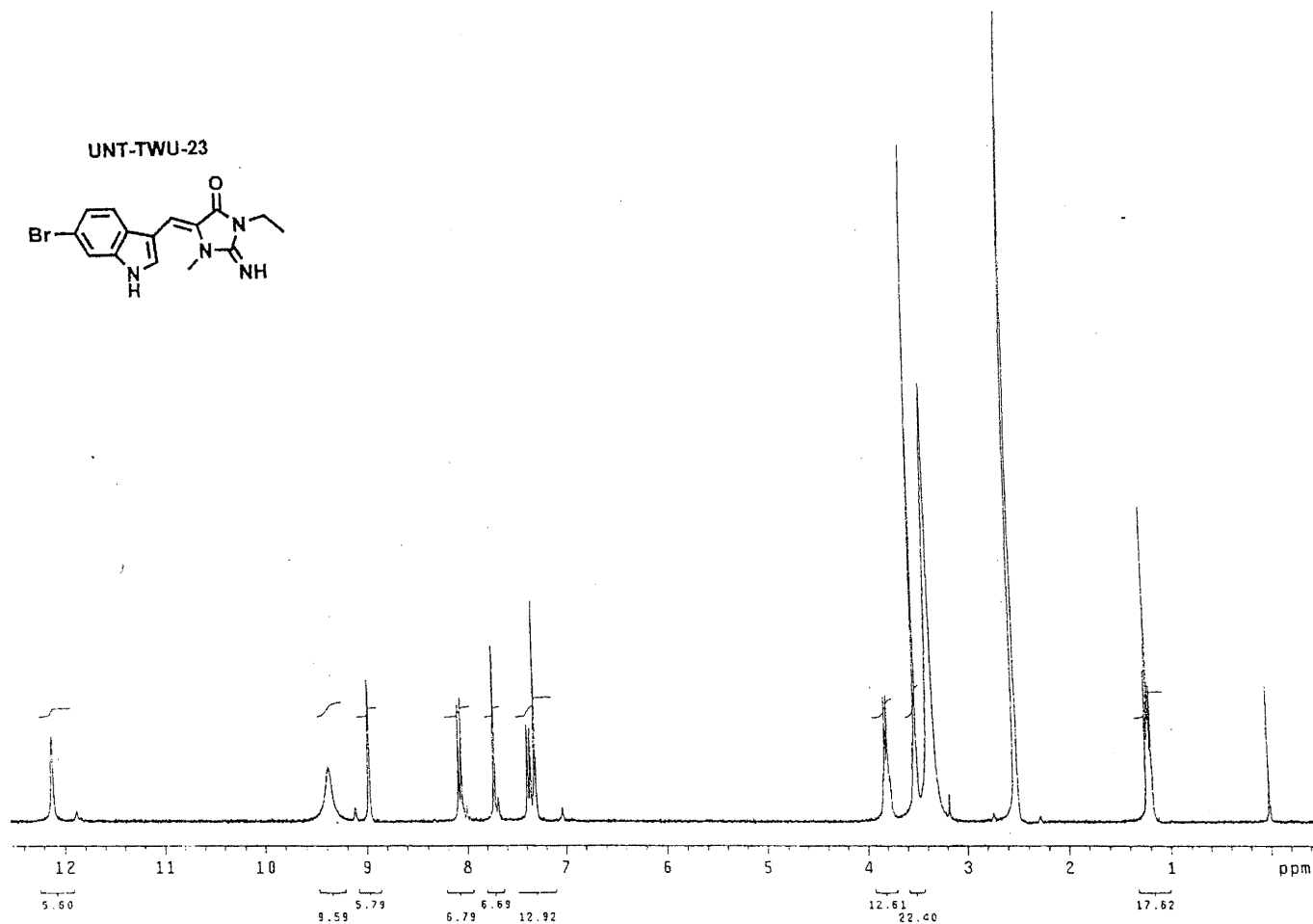
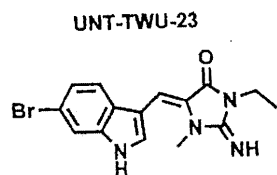




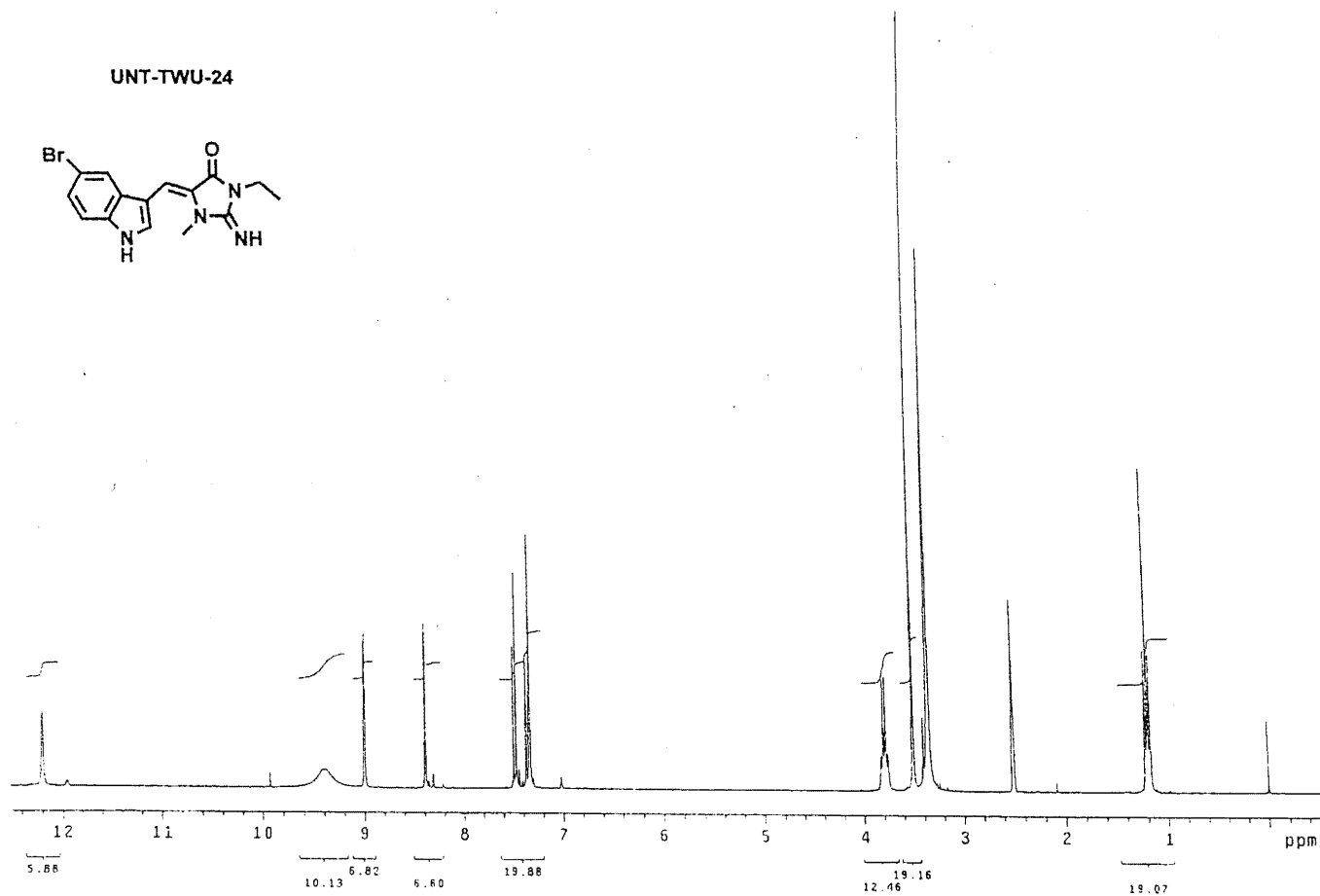
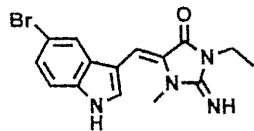




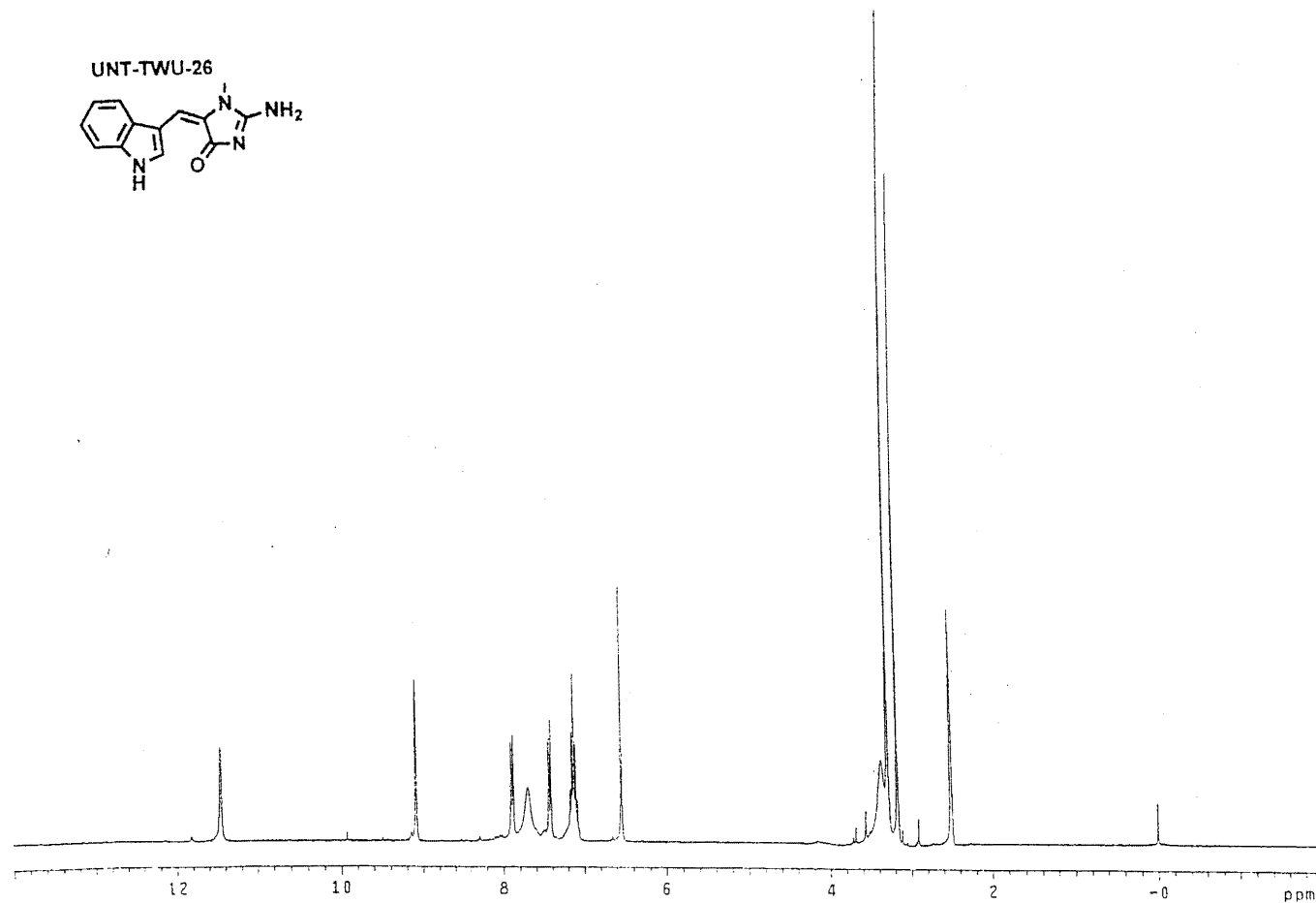
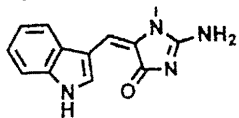




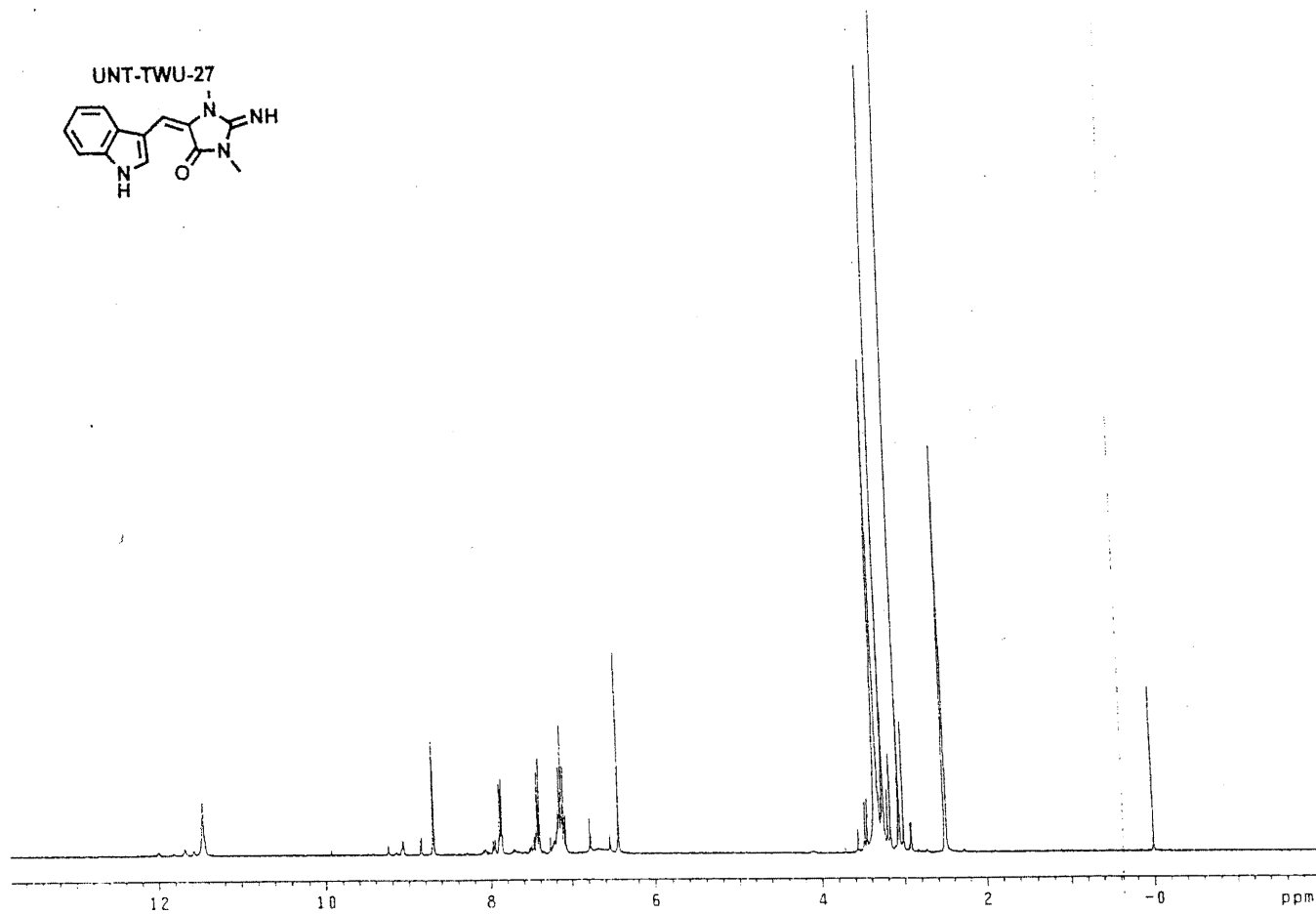
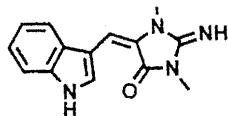
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UNT-TWU-27



UNT-TWU-28

