Isolation and Characterization of Steroidal Alkaloids from *Buxus macowanii* Using Chromatographic and Spectroscopic Methods

by

Manal Almalki

A Thesis submitted to the Faculty of Graduate Studies of

The University of Manitoba

in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

Department of Chemistry

University of Manitoba

Winnipeg

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ABSTRACT

This thesis describes the isolation and characterization of four steroidal alkaloids from *Buxus macowanii*.

Phytochemical investigation of *Buxus macowanii* resulted in the isolation of two novel steroidal alkaloids, N_b-demethyl-6-deoxy-16-acetoxy O^2-natafuranamin (**112**), and 6-deoxy-16-acetoxy O^{10}-natafuranamin (**113**) alongside two known steroidal alkaloids, cycloprotobuxine-D (**114**), and cycloprotobuxine-F (**115**). Compounds **114** and **115** have been isolated for the first time from *B. macowanii.* Structure of compounds **112-115** was elucidated with aid of UV, IR, mass, and 1D and 2D NMR spectroscopy. These compounds showed different level of anti-AChE activity. Among all the isolates, compound 112 was found to be significantly active against AChE with an IC_{50} value of 4.7 μM. The bioactivity of this new compound nearly comparable to those of huperzine (IC_{50} = 1.7 μM) and O^2-natafuranamine (IC_{50} = 3.0 μM).

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I would like to express my gratefulness to my supervisor, Dr. Athar Ata for his support, direction, and suggestions that allowed this thesis to be possible. I would also like to thank all the members of the Department of Chemistry at the University of Winnipeg, and University of Manitoba for their assistance. I appreciate the technical support provided by Mr. Ramin Vakili. I thank the examining committee members for spending time in evaluating my thesis. Also, I value the encouragement given by my colleagues in Dr. Ata's Lab. I would like to express my thanks to all my family members for their support and patience during this course of work, especially my husband Ali, my kids, Zaid and Rafaa, my mother, and my father.

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GLOSSARY

^{13}C-NMR	Carbon Nuclear Magnetic Resonance Spectroscopy: Provides information about the electronic environment of ^{13}C- atoms in the molecule.
^{1}H-NMR	Proton Nuclear Magnetic Resonance Spectroscopy: Provides information about the electronic environment of ^{1}H- atoms in the molecule.
APT	Attached Proton Test: Used to differentiate between methyl, methylene, and quaternary carbons.
DEPT	Distortionless Enhancement by Polarization Transfer: Used to differentiate between all protonated carbons, methyl, methylene, and methine and quaternary carbons.
HSQC	The Heteronuclear Single Quantum Coherence: Shows ^{1}H/ ^{13}C one-bond correlation of proton bearing carbons.
HMBC	The Heteronuclear Multiple-Bond Correlation Spectroscopy: Displays the heteronuclear correlations over 2–4 bonds.
COSY	The Correlation Spectroscopy: Provides geminal and vicinal couplings of protons.
TOCSY	The Correlation Spectroscopy: Provides long-range couplings of protons.
NOESY	Nuclear Overhauser Effect Spectroscopy: Displays proton-proton correlation through space.

$CDCl_3$	Deuterated Chloroform: Solvent used for NMR spectroscopy.
CD_3 CO CD_3	Deuterated Acetone : Solvent used for NMR spectroscopy.
ACh	Acetylcholine: An important neurotransmitter in the central nervous system.
AChE	Acetylcholinesterase: An enzyme that catalyze the degradation of acetylcholine.
AD	Alzheimer Disease: The most common neurodegenerative disorder.
IC_{50}	The concentration required to inhibit 50% of the enzyme function.
HR-TOf-MS	High- Resolution Time of Flight Mass Spectrometry: A method used to determine the ion's mass to charge ratio through time determination, providing the molecular mass.
LR-EL-MS	Low-Resolution Electron Impact Mass Spectrometry: A method used to determine the mass molecular.
$[M^+ +H]$	Molecular Ion Peak: The peak that presents the highest mass to charge ratio of the molecule.
IR	Infrared Spectroscopy: Utilized to determine the functional groups in the organic compounds based on their absorption.
UV	Ultraviolet Spectroscopy: Provides information about chromospheres.
TLC	Thin Layer Chromatography

CHAPTER 1

NATURAL PRODUCT CHEMISTRY

1.1 INTRODUCTION

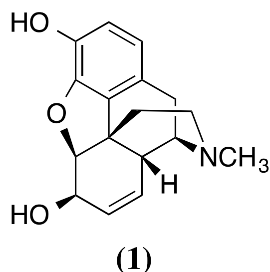
Since prehistoric times, different parts of plants have been used to treat various health problems. These medicinal uses of plant resulted in the birth of natural product chemistry.[1] Natural products are organic compounds, produced by different living organisms including, plants, microbes, and marine organisms. These compounds are also known as secondary metabolites. There are two types of metabolites produced by plants and marine organisms: (i) primary metabolites, and (ii) secondary metabolites.[2] Primary metabolites are simple compounds required for normal function and are the building blocks of life. Secondary metabolites are produced by primary metabolites and do not play an important role in the growth, development, or reproduction of living organisms.[2] However, secondary metabolites help organisms in surviving nature.[3] For example, secondary metabolites have been known to play an important role in plant defense against herbivores. These metabolites exhibit various biological activities making them an ideal source for the drug discovery program. The development of modern chromatography and spectroscopic techniques helped organic chemist to isolate and characterize secondary metabolites. These metabolites are used as a source of lead compounds for drug discovery program due to their enormous structural diversity, which is difficult to obtain by combinatorial chemistry or traditional synthetic approaches.

1.1.1 BRIEF HISTORY ABOUT NATURAL PRODUCT

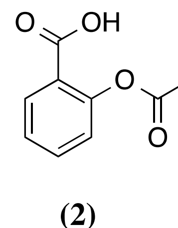
Traditional people have utilized plants as medicinal agents for thousands of years. For instance, the opium plant was used to relieve pain and fever. These medicinal uses of plants are thoroughly recorded in literature by numerous cultures in all eras. The oldest and best record of natural product based medicine dates back to 1500 BCE in Mesopotamia; the Ebers Papyrus documented over 700 drugs derived mostly from plants. Another example of these records is the Chinese 'Materia Medica', which documents 52 prescriptions dating as far back as 1100 BCE. Similarly, the ancient Indian Ayurvedic medicinal system is recorded earlier than 1000 BCE. The use of herbal remedies in Roman societies has also been documented.[4]

The aforementioned documents resulted in the isolation of morphine (**1**), a painkiller from opium plant by the pharmacist Friedrich Serturner in 1805.[5] The significance of this discovery subsequently resulted in the synthesis of this compound. In 1826, morphine became the first pure compound based on natural products to be available commercially by Merck.[4] Even with the development of the anesthesia system in the last few years, morphine persists to be the most common analgesic drug in clinics. This discovery then led to the discovery of aspirin (**2**), the first semi-synthetic pure drug based on a natural product by Bayer in 1899.[4] Currently, aspirin is the gold standard agent for the primary and secondary prevention of thromboembolic diseases.[6] These discoveries, in conjunction with the discovery of a number of other lead bioactive compounds, resulted in the establishment of natural products chemistry, a new class of

organic chemistry. This new branch still plays a significant role by contributing lead compounds to the drug discovery program.



(1)



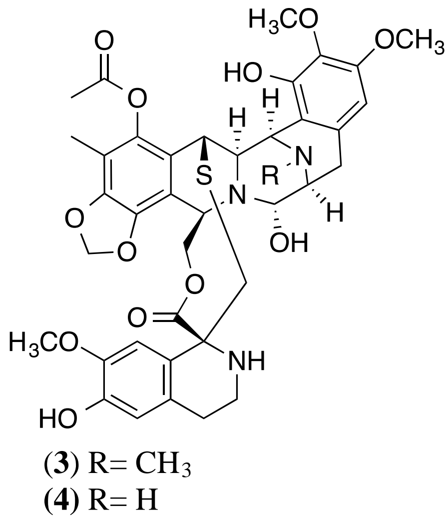
(2)

Although researchers have isolated natural products from various sources including, plants, microbes, and marine organisms, plants are considered as one of the major sources of bioactive compounds as evidence in the literature. The literature survey indicates that nearly 25% of drugs are produced from plants and 16% by microbial and animal sources.[6] There are over 250,000 species of higher plants present in the world, and less than 5% of these plants have been investigated for the discovery of biologically active compounds and their potential in pharmaceutical applications.[7]

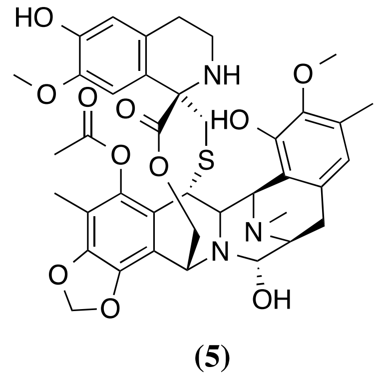
Marine organisms have also provided lead bioactive compounds with novel carbon skeletons. For instance, ecteinascidin-743 (**3**), (trade name yondelis), the first marine derived anticancer compound, was isolated from the Caribbean tunicate *Ecteinascidia turbinate.* In July 2007, ecteinascidin-743 was the first refractory soft-tissue sarcomas drug approved by the European Commission. Another example is ecteinascidin-729 (**4**), *N*-demethyl analogue of ecteinascidin-743 isolated from the same marine organism, was equally potent against soft tissue cancer. In October 2007, trabectedin (**5**) another marine derived natural product, was approved as anti-cancer pharmaceutical by the European Union.[8,9]

Natural products of fungal origin have also shown potential as therapeutic agents. For instance, FTY720 (**6**) fingolimod (trade name Gilenya, Novartis), derived from the fungus *Isaria sinclairii,* exhibits activity against chronic rejection, and tumor growth.[10] Another example of fungal natural product is penicillin (**7**), an antibiotic isolated from the fungus *Penicillium notatum,* was discovered in 1928 by Alexander Fleming.[11] The discovery of natural products with antibiotic activity was at its peak between the 1940s to 1950s.[12] Although natural products have been produced by various organisms, plant natural products still play an important role in the discovery of new biomedical agents for the health care system.

Another aspect of natural product based drug discovery involves the structural modification of bioactive natural products using biosynthesis or chemical synthesis approaches. For example, dehydrozingerone (**8**), a phenolic compound isolated from ginger (*Zingiber officinale*) rhizomes, exhibits antioxidant and anti-tyrosinase activities.[13] This compound is also used to treat Alzheimer's disease (AD).[14] Dehydrozingerone derivatives were synthesized in order to enhance antioxidant and anti-tyrosinase properties. Out of the twenty-nine derivatives synthesized, six compounds (**9-14)** display enhanced antioxidant activity compared to the parent compound (**8**) and rest of the analogue. Moreover, compound (**12)** inhibits Fe^{2+}-induced lipid peroxidation and compound (**15)** shows anti-tyrosinase activity. The increased antioxidant potential is reported to the number and placement of the hydroxyl group on the aromatic ring as well as the introduction of a double bond between C-3, and C-4 carbons. These compounds exhibit significantly higher radical scavenging activity than the standard antioxidant α-tocopherol and ascorbic acid.[13]



(**3**) R= CH_3
(**4**) R= H



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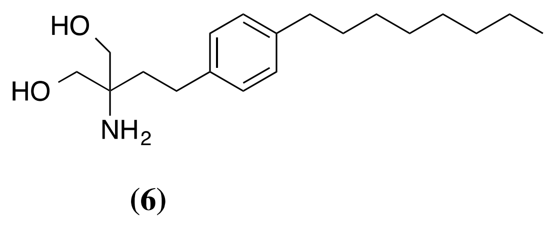

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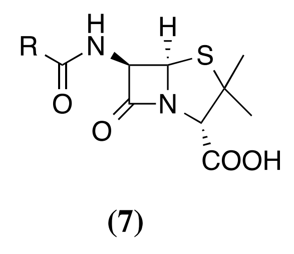

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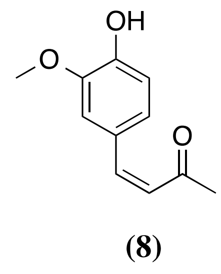

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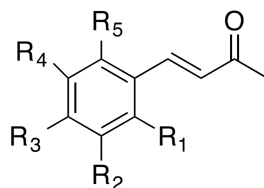


(**9**) R1= H, R2= OCH3, R3=OH, R4=H, R5=H

(**10**) R1=OH, R2=H, R3=H, R4=OCH3, R5=H

(**11**) R1=H, R2=OCH3, R3=OH, R4=OCH3, R5=H

(**12**) R1=OH, R2=OH, R3=H, R4=H, R5=H

(**13**) R1=OH, R2=H, R3=H, R4=OH, R5=H

(**14**) R1=H, R2=OH, R3=OH, R4=H, R5=H

(**15**) R1=OH, R2=H, R3=OH, R4=H, R5=H

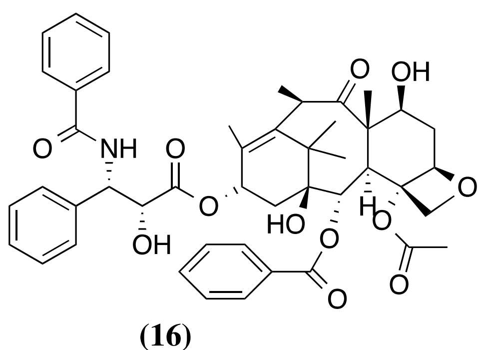
1.1.2 NATURAL PRODUCT IN DRUG DISCOVERY AND THEIR PHARMACEUTICAL APPLICATIONS

Natural products and their derivatives have been used as a major source of pharmaceutical agents for centuries. Approximately 49% of 877 new chemical entities discovered between 1981 and 2002 are either natural products, or natural products based synthetic compounds.[1,5] However, the natural products based drugs discovery program in the pharmaceutical industry have shown a decline within the last two decades due to three main reasons: (i) The long period of time between the initial discovery of a bioactive component in the crude extract phase and being commercially available in the market. (ii) The admittance of high throughput screening (HTS) which allowed chemists to assay

millions of chemical molecule and identify their bioactive components against specific targets. (iii) The advancement in combinatorial chemistry (CC), a synthetic method, which also allows researchers to generate a large number of compounds and identify the valuable constituent in these compounds. Although the aim of all these substituted strategies was to provide molecules with high chemical structural diversity and specific biological activity, these methods have not provided result in some therapeutic areas such as immunosuppression, anti-infective, and metabolic ailments.[16] As a result of this, it was realized that natural products are the only source of novel bioactive components in the drug discovery program with high biochemical properties such as molecular mass, large number of chiral centres, and ring topology. These high molecular properties alongside advanced technologies helped to renew the interest into natural products in drug discovery.[15]

Nowadays, approximately 30% of drugs in global markets are of natural product origin. To discover and produce new chemical entities, pharmaceutical companies have to spend approximately US $350 million.[17] It is documented that 87% of all human ailments have been treated by natural product based drugs including antibacterial, anticancer, anticoagulant, anti-parasitic, and immunosuppressant agents.[18] The global ethical drug market estimates that natural products isolated from living organisms were responsible for generating 24%, 26% and 40% of total revenue during the years 2000, 2001, and 2002, respectively.[18] Two classes of cancer chemotherapeutic agents, taxanes; paclitaxel (16) and docetaxel (17), and the camptothecin derivatives; irinotecan (18) and topotecan (19) were responsible for $3 billion sales in 2002, which is one-third of the total anticancer drug sales in the worldwide. Paclitaxel (**16**) alone was ranked at 25th in

2000 after generating sales of an astounding $1.6 billion in that year. In addition to these biochemical entities which are commercially available in the market, there are nearly 100 new bioactive compounds from natural sources under clinical trial such as combretastatin-A4 phosphate (**20**) which shows activity in disrupting tumor vessels, and arteether (**21**) as a potential anti-malarial agent.[19,20]



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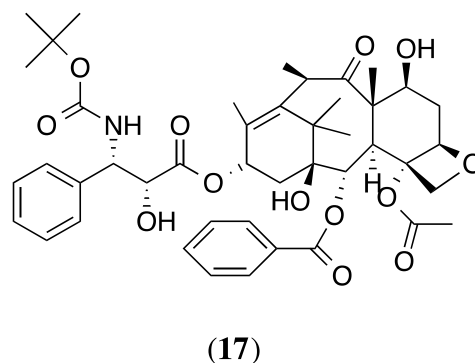
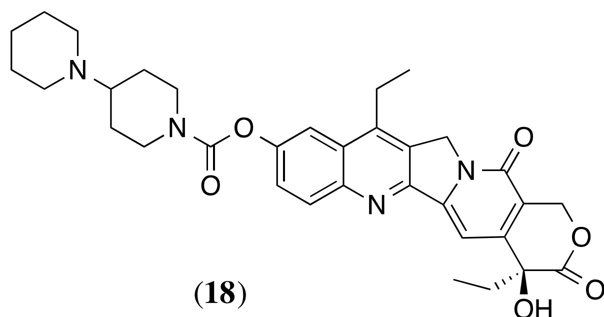
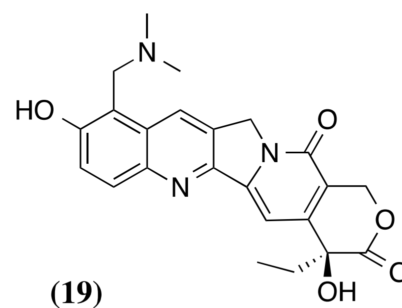


(**17**)



(**18**)



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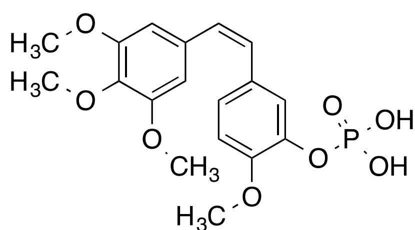
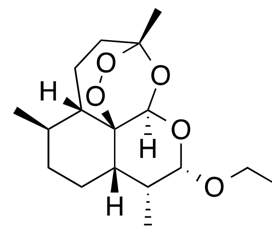


(**20**)



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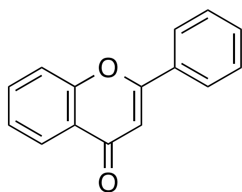
1.2 TYPES OF NATURAL PRODUCTS

The secondary metabolites with their small molecular weight (< 3,000 Daltons) can be categorized into different groups based on their structural features and biosynthetic origin such as flavonoids, terpenoids, steroids, and alkaloids.[21] It is worthwhile to mention that natural products are not classified based on just one key intermediate building block involved in their biosynthetic pathway, as most natural products utilize more than one basic building block.[2]

1.2.1 FLAVONOIDS

Flavonoids are widely spread in the plant kingdom, and are produced by various parts of plants, however they are specifically abundant in the photosynthesizing plant cells. Flavonoids are heterocyclic organic compounds derived from 2-phenylchromone a parent compound. Flavonoids can be classified based on their chemical nature such as degree of hydroxylation, degree of polymerization, and the position of different substituents on A, B, and C, the three phenolic rings of their carbon skeleton. Flavonoids are classified into six main structural classes including flavone (**22**), chalcone (**23**), flavonol (**24**), flavan-3-ol (**25**), flavanone (**26**), flavolan (**27**).[22-24]

Flavonoids are an important component of human diet, as they exist in various food sources including fruits, vegetables, chocolate, and teas. These compounds exhibit various biological activities and help to protect us from various infectious diseases including, sores, wound infections, acne, and urinary tract infections. Most of the reported flavonoids exhibit anti-bacterial activity.[22-24] Similarly aromatic compounds closely related to flavonoids are also reported to exhibit anti-bacterial activity against Gram-positive and Gram-negative bacteria. For example, panduratin A (**28**), a natural phenolic compound isolated from the rhizome of fingerroot *(Boesenbergia rotunda)*, exhibits anti-bacterial activity against Gram-positive bacteria. Other examples of anti-bacterial flavonoids include isobavachalcone (**29**), bartericin (**30**).[23,25] The anti-bacterial activity of compounds (**28-30**) is listed in **Table 1.1**.



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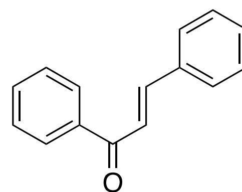
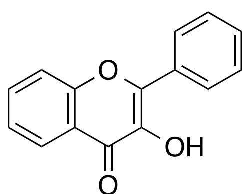


(**23**)



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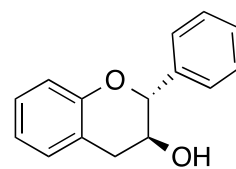
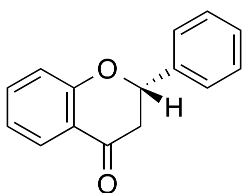
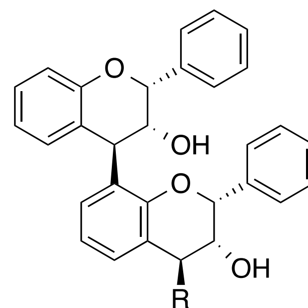


(**25**)



(**26**)

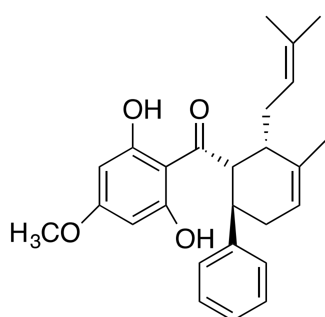


(**27**)

Table 1.1. Anti-bacterial activity of flavonoids (**28**-**30**).

Flavonoids	Gram-positive MIC (μg/ml)	Gram-negative MIC (μg/ml)
Panduratin A (**28**)	0.06-2.0	-
Isobavachalcone (**29**)	0.3-0.6	0.3
Bartericin A (**30**)	0.6-2.4	0.3

MIC represents minimum inhibitory concentration.



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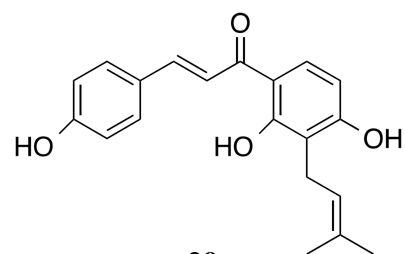

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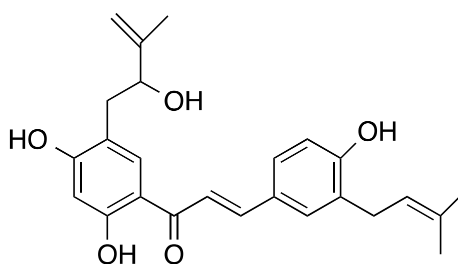

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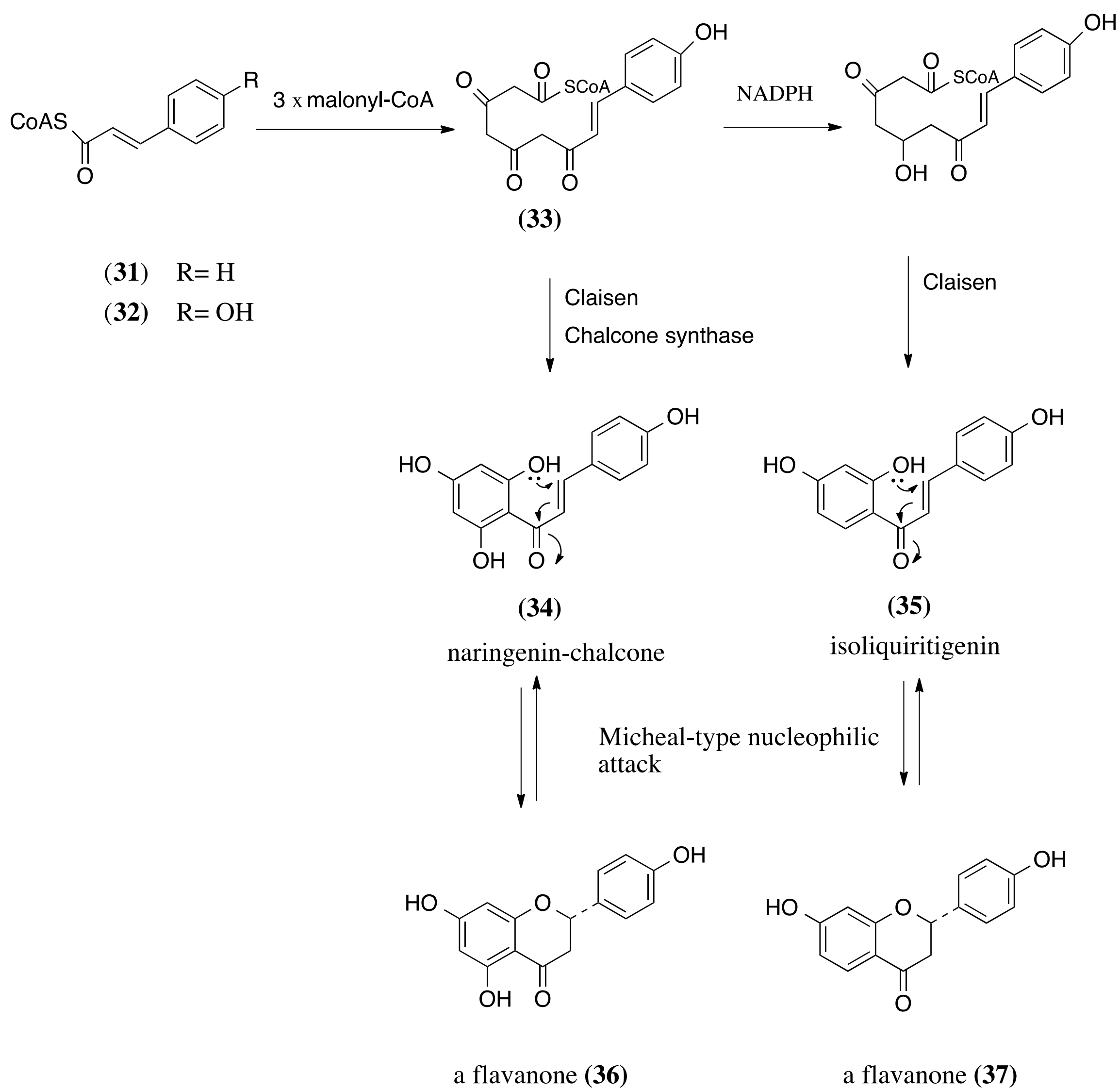
Flavonoids are biosynthesized in plants using cinnamoyl-CoA (**31**) as a starting precursor. The carbon chain of 4-hydroxycinnamoyl-CoA (**32**) is extended by the reaction of three molecules of malonyl-CoA, to produce a polyketied (**33**). The polyketied **33** is processed by two different ways to produce chalcones, precursors of flavonoids in plants. First, a claisen type cyclization is utilized in the presence of chalcone synthase, to form naringenin-chalcone (**34**). Second, a polyketide is reduced and undergoes a claissen reaction to form isoliquiritigenin (**35**). These products undergo a Michael-type nucleophilic attack of a phenol group on the α,β-unsaturated ketone to form flavanone (**36**-**37**), respectively (**Scheme 1.1**).[26]

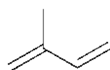


Scheme 1.1. Biosynthesis of flavonoid from cinnamoyl-CoA.

1.2.2 TERPENOIDS

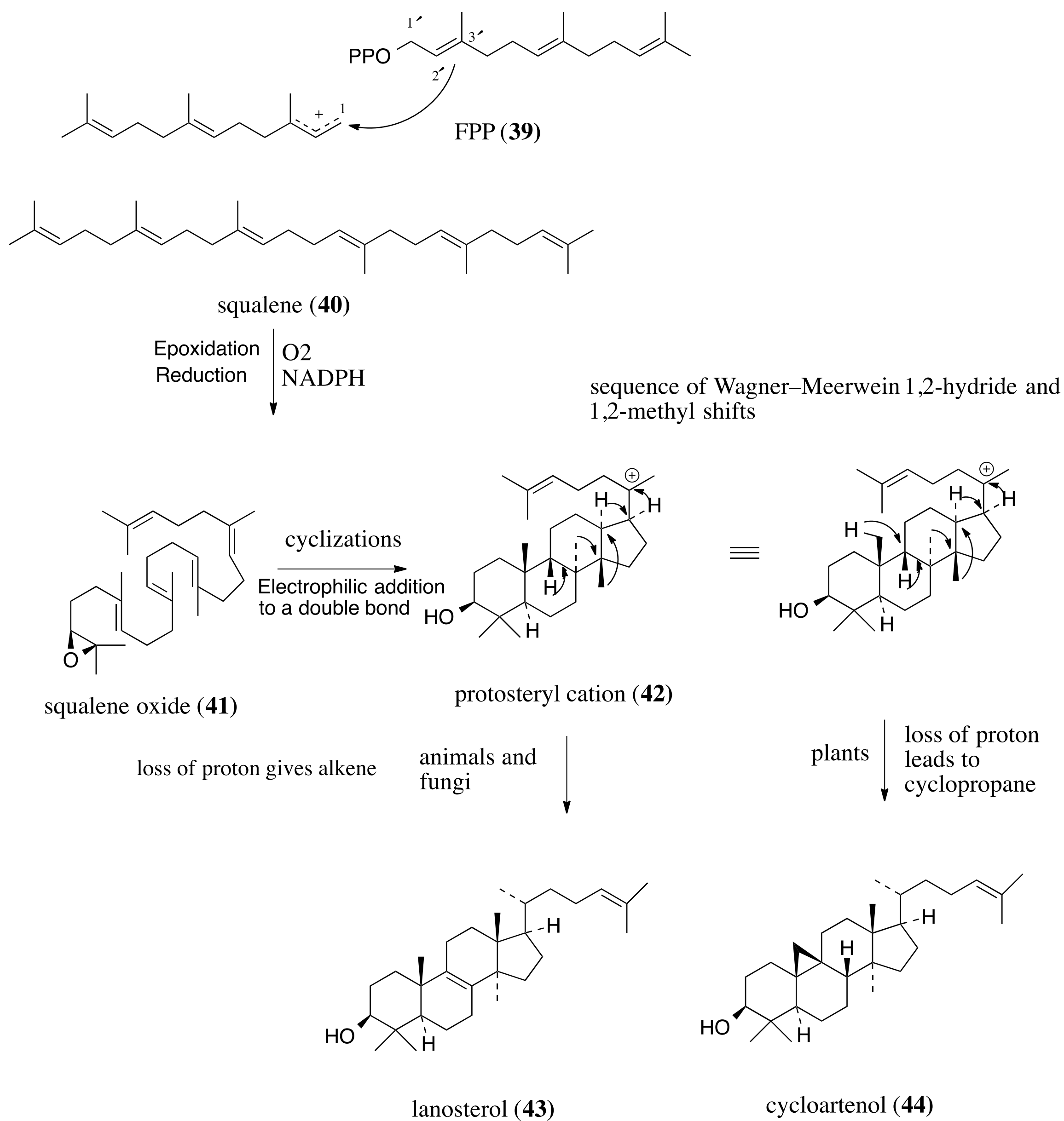
Terpenoids, also known as terpenes, represent a large group of natural products and are used to treat various health problems. Terpenoids are reported to exhibit various biological activities including antiviral, antibacterial etc. These compounds are used as flavors, food additives, and cosmetics.[27]

In nature, terpenoids are produced using isoprene unit (**38**) as a building block. Terpenoids are classified as monoterpenoids, sesquiterpenoids, diterpenoids, and trieterpenes based on the presence of two, three, four, and six isoprene units in their skeletons, respectively.[27]



38

Terpenoids are biosynthesized by coupling of isoprene units. The C-30 triterpenoids are formed by the tail-tail coupling of two farnesyl pyrophosphate FPP (**39**) to afford squalene (**40**). Squalene undergoes oxidation to yield squalene-2,3-oxide (**41**). A series of cyclization reactions occur to produce the polycyclic triterpene structure, protosteryl cation (**42**). The polycyclic triterpene undergoes a sequence of Wagner–Meerwein migrations of methyls and hydrides to produce lanosterol (**43**) in animals and fungi and cycloartenol (**44**) in plants (**Scheme 1.2**).[26] These compounds are considered as biosynthetic precursors to various terpenoids. For instance, cycloartenol (**44**) is assumed to be the biosynthetic intermediate for *Buxus* alkaloids, as discussed on page 29.

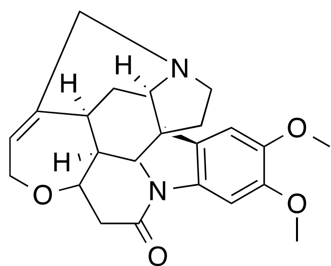


Scheme 1.2. Biosynthesis of triterpenoids from squalene.

1.2.3 ALKALOIDS

Alkaloids contain nitrogen in their structure, which are also widely present in nature. These compounds contain one or more nitrogen atoms in the form of primary, secondary, tertiary, or quaternary amines.[26] During 1817-1821, pharmacists isolated a large number of alkaloids such as brucine (**45**), quinine (**46**), caffeine (**47**), and veratrine (**48**).[28]

There are many possible ways to classify alkaloids into different groups. One such method is on the basis of amino acids. One group of alkaloids derived from an amino acid precursor that presents a part of their skeleton such as alkaloids derived from ornithine, lysine, nicotinic acid, tyrosine, tryptophan, anthranilic acid, and histidine. In this type of alkaloids, the skeleton of an amino acid precursor remains intact but the carboxylic acid moiety is lost by decarboxylation process.[26] These alkaloids generally have a pyrrolidine ring linked to their structure. One notable example includes cocaine, which is derived by the decarboxylation of L-ornithine (**Scheme1.3**).[29] L-ornithine (**49**) is produced in animals from L-arginine, while it is synthesized in plants from L-glutamic acid.[26] After producing putrescin (**50**), it undergoes further modification to yield a *N*-methyl Δ^1-pyrrolinium cation (**51**). After several reactions including oxidation and condensation, this affords ecgonone CoA ester (**52**), which on hydrolysis of CoA moiety yields methyl ester, methylecgonone (**53**), a precursor of cocaine. This precursor on reduction and benzoylation affords cocaine (**54**) .[29]



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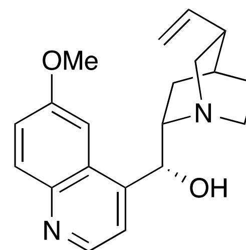

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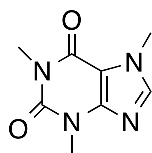

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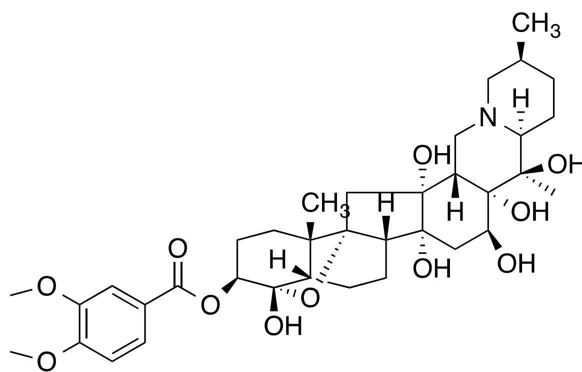

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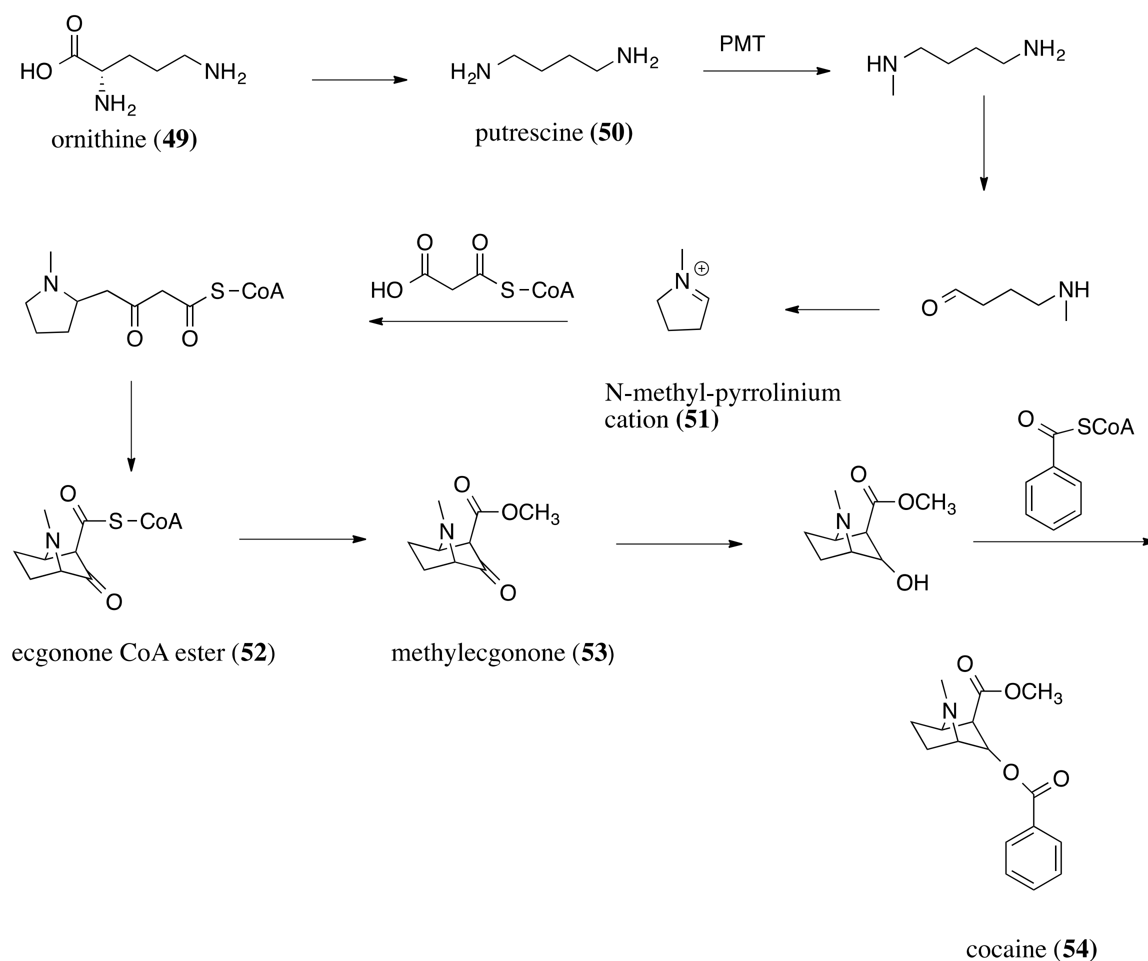
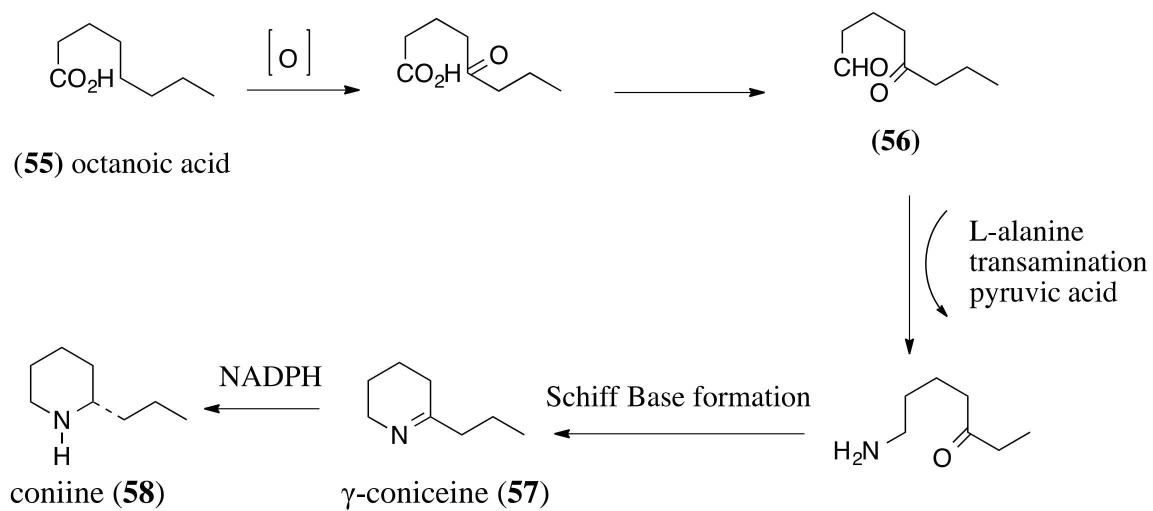


Scheme 1.3. Biosynthesis of alkaloid cocaine from L-ornithine

Alkaloids that are derived from non-amino acid precursors, obtain their nitrogen atom from amino acid in a later stage of their biosynthesis by transamination reactions. For example, γ-coniceine and coniine are derived from octanoic acid (**55**), which undergoes successive oxidation and reduction reactions to afford a ketoaldehyde intermediate (**56**). This ketoaldehyde by a transamination reaction using L-alanine produce γ-coniceine (**57**) and coniine (**58**) (**Scheme1.4**).[26]



Scheme 1.4. Biosynthesis of coniine and γ-coniceine by transamination reaction.

1.2.4 STEROIDAL ALKALOIDS

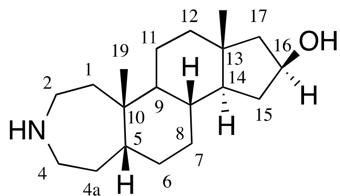
Steroidal Alkaloids are important class of organic compounds, which are obtained from various natural sources such as animals, marine organisms, and many species of plants. The basic chemical structure of steroidal alkaloids is cyclopentenophenanthrene with nitrogen atom in the ring or in the side chain of the molecule. Steroidal alkaloids display important biological and pharmacological properties including antimicrobial and anticancer activates etc.[30] Moreover, they are found to be active in the treatment of Alzheimer's disease.[31] Steroidal alkaloids containing glycosidic moiety (saponin) are also reported to have surface activity and haemolytic properties.[26]

Steroidal alkaloids are mainly divided into three groups based on their natural origin: (i) steroidal alkaloids from animal sources, (ii) from marine sources, and (iii) from plants. Steroidal alkaloids of plant origin are classified as *Solanum* alkaloids, *Apocynaceae* alkaloid*s*, and *Buxus* alkaloids.[32]

Steroidal alkaloids from animal sources are classified into two classes: (i) *Salamandra* alkaloids and (ii) *Phyllobates* alkaloids. There are two subclasses of *Salamandra* alkaloids; samanine (**59**) and samandarine (**60**). The major steroidal alkaloids of *Phyllobates* are batrachotoxinine-A (**61**) and batrachotoxin (**62**). Some amphibians are capable of synthesizing these steroidal alkaloids to protect against fungal and bacterial infection.[33]

Various steroidal alkaloids have been isolated from marine organisms. Some of these compounds have the pregnane type structure. Two representative examples of these

steroidal alkaloids are plakinamine-A (**63**) and plakinamine-B (**64**) extracted from marine sponge of the genus *Plakina*.[34]



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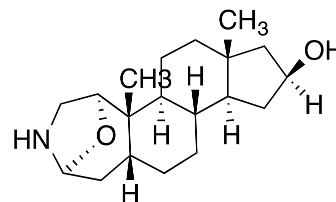

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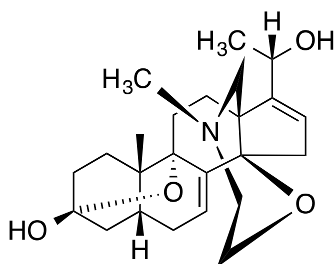

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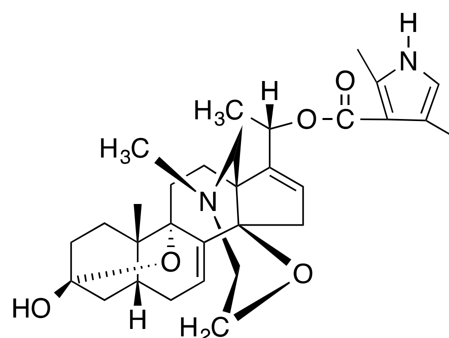
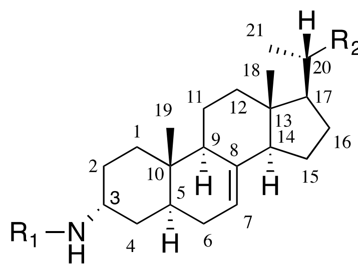
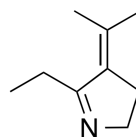
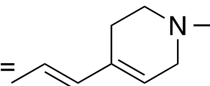


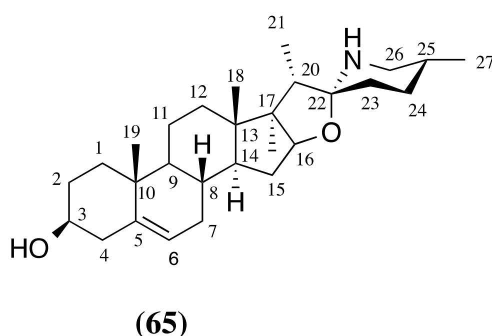
(62)



(63) R_1= H , R2= 

(64) R_1= CH3, R2= 

The family *Solanaceae* is a rich source of steroidal alkaloids. Steroidal alkaloids isolated from this family occur as glycosides, the aglycones of which possess the C_{27} carbon skeleton of cholestane, and are divided into five groups based on their chemical structure: (i) spirosolanes, (ii) epiminocholestanes, (iii) solanidanes, (iv) solanocapsine, (v) 3-aminospirostanes. Solasodine (**65**), solacongestidine (**66**), solanidine (**67**), solanocapsine (**68**), and jurubidine (**69**), are representative examples of steroidal alkaloids spirosolanes, epiminocholestanes, solanidanes, solanocapsine, and 3-aminospirostanes, respectively.[35]



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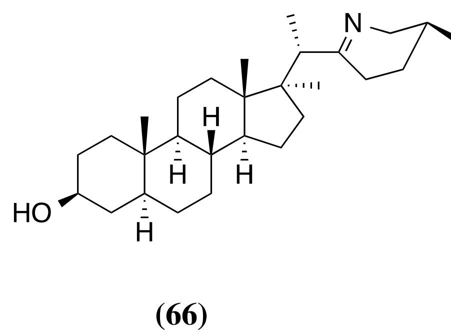

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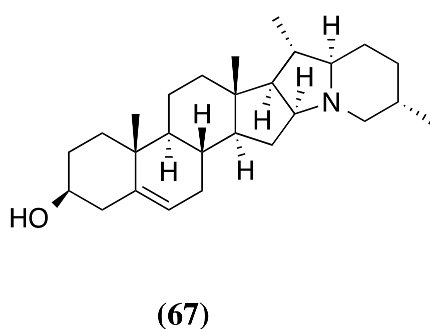

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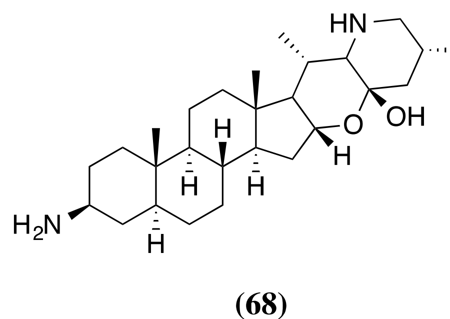

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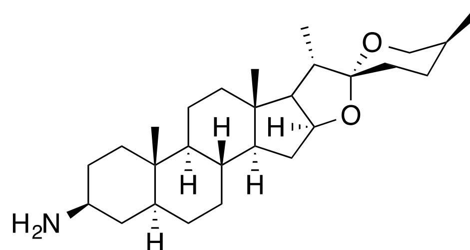
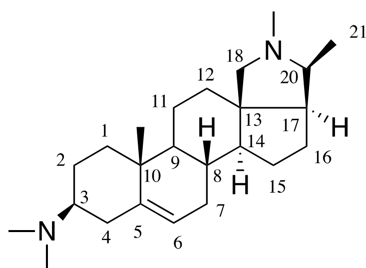


(69)

The family *Apocynaceae* is also considered as a rich source of steroidal alkaloids. These steroidal alkaloids are of two types. (i) conanine type, and (ii) non-conanine type. Conessine (**70**) and funtumine (**71**) isolated from *Funtumia* genera are examples of these two types, respectively.[32,37]



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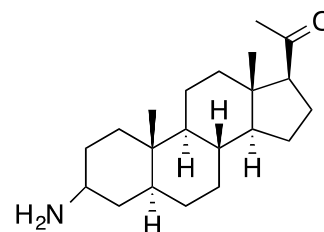
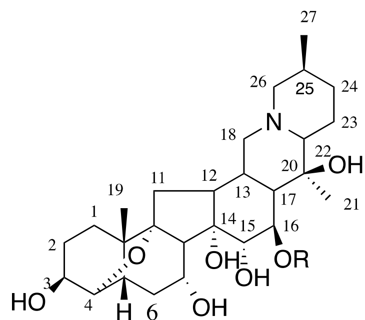


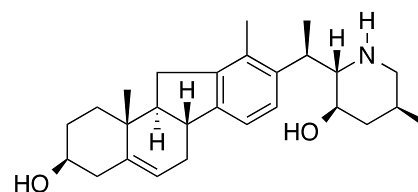
(71)

In China, people have been using the roots and rhizomes of several *Veratrum* species, to treat aphasia arising from apoplexy, wind-type dysentery, jaundice, scabies and chronic malaria for centuries. Also, they have been using the bulbs of many *Fritillaria* species as an anti-tussive and anti-asthmatic for more than 2000 years.[38] Steroidal alkaloids, which are isolated from the family *Liliaceae,* contain a C_{27} cholestane carbon skeleton with five or six carbocyclic or heterocyclic rings and are classified into two groups: (i) isosteroidal alkaloids, and (ii) steroidal alkaloids based on

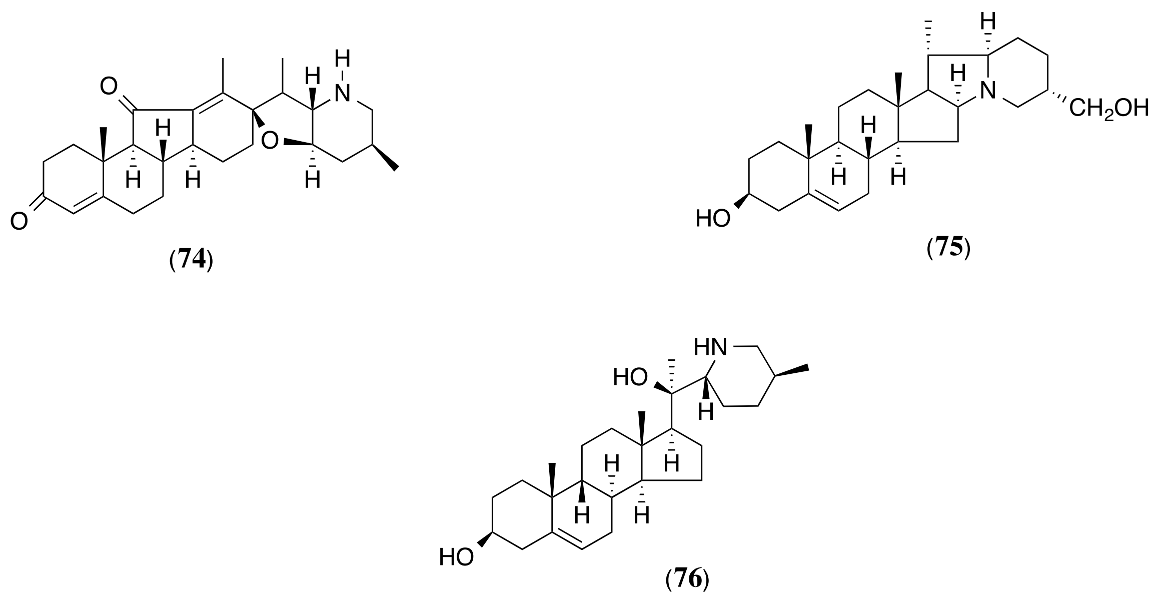
the carbon skeleton. The former class belongs to *Veratrum* steroidal alkaloids, characterised by a C-nor-D-homo-[14(13→12)-*abeo*] ring and can be divided into subclasses based on the linkage patterns between rings E and F into cevanine, veratramine, and jervine. The last type contains six-membered C-ring and five-membered D-ring and can be divided into solanidine and verazine types depending on the presence of nitrogen in the rings.[39] Verussurinine (**72**), 20-isoveratramine (**73**), jervinone (**74**), camtschatcanidine (**75**), and stenophylline B (**76**) are representative examples of steroidal alkaloids of cevanine, veratramine, jervine, solanidine, and verazine, respectively.



(**72**) R= Methybutyroyl

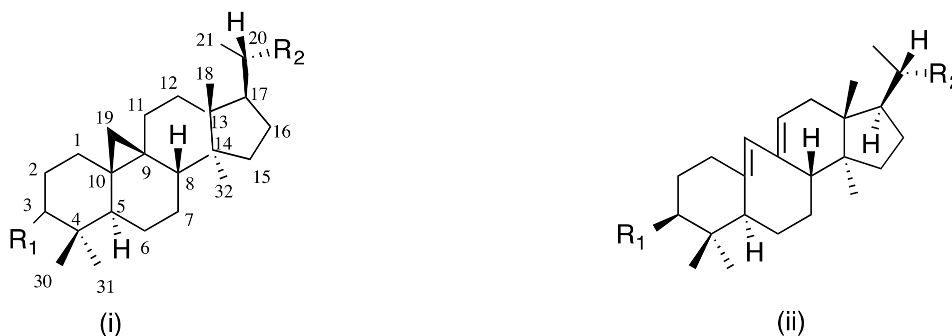


(**73**)

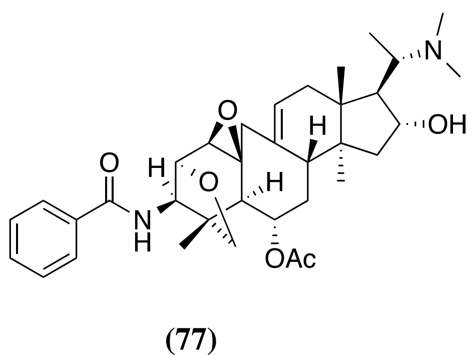


Buxus alkaloids have a unique steroid-triterpenoid pregnane type structure with C-4 methyl groups, a 9*β*,10*β*-cycloartenol system, and a degraded C-20 side Chain.[40,41] There are two classes of *Buxus* alkaloids: (i) 9*β*,10*β*-*cyclo*-4,4,14α-trimethyl-5-α-pregnane system, and (ii) 9(10→19) *abeo* 4,4,14α-trimethyl-5-α-pregnane system. Both of these classes can easily be identified with the aid of NMR spectroscopy. Alkaloids of the class (i) series exhibit a pair of AB doublet in the up-field region at δ 0.1-0.5 (J = 4.0 Hz) due to C-19 cyclopropyl methylene protons in their ^{1}H-NMR spectrum. Alkaloids of class (ii) series show signals in the olefinic range due to the C-11 and C-19 methine protons in their ^{1}H-NMR spectrum. Compounds of class (ii) containing 9(10→19) *abeo* diene system exhibit UV absorption at 238 and 245 nm with shoulders at 228 and 252 nm in the UV spectrum. Based on these two spectral aspects, it is simple to differentiate between the two classes of alkaloids.[40]

Buxus alkaloids exhibit various biological properties such as anti-HIV, anti-TB, and enzyme inhibitory activities.[41] For example, the steroidal alkaloids, O^{2}-natafuranamine (**77**), O^{10}-natafuranamine (**78**), and buxafuranamide (**79**), isolated from *B. natalensis* show strong acetylcholinesterase inhibitory activity.[42] Another example of AChE inhibition is the steroidal alkaloids isolated from *B. hyrcana*, spirofornabuxine (**80**) and buxamine B (**81**).[43-45] Also buxakashmiramine (**82**), which isolated from *B. papillosa,* exhibits anti-AChE activity.[46]



R_1 and R_2 represent different amino or keto functionalties



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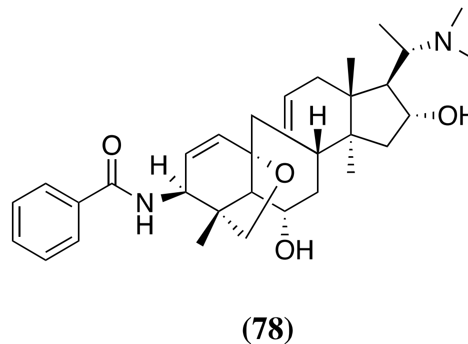

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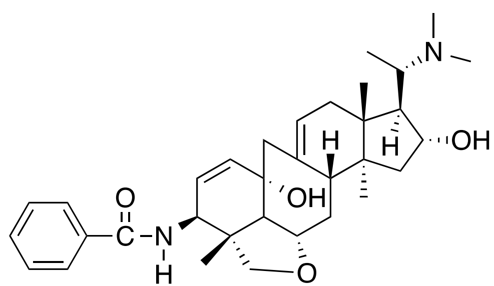

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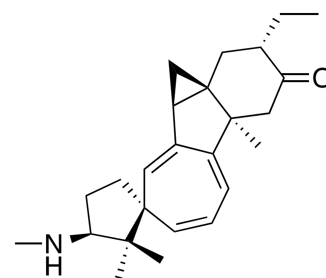

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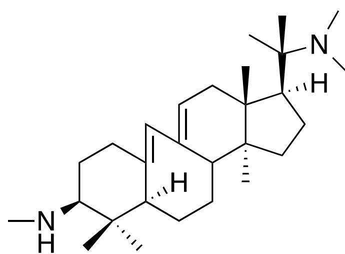

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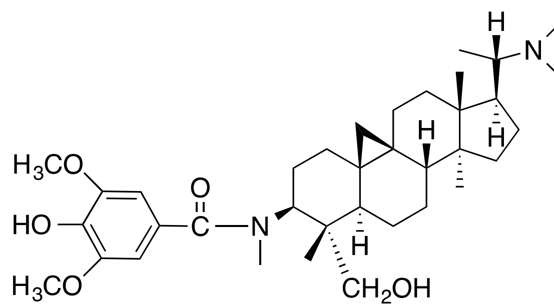


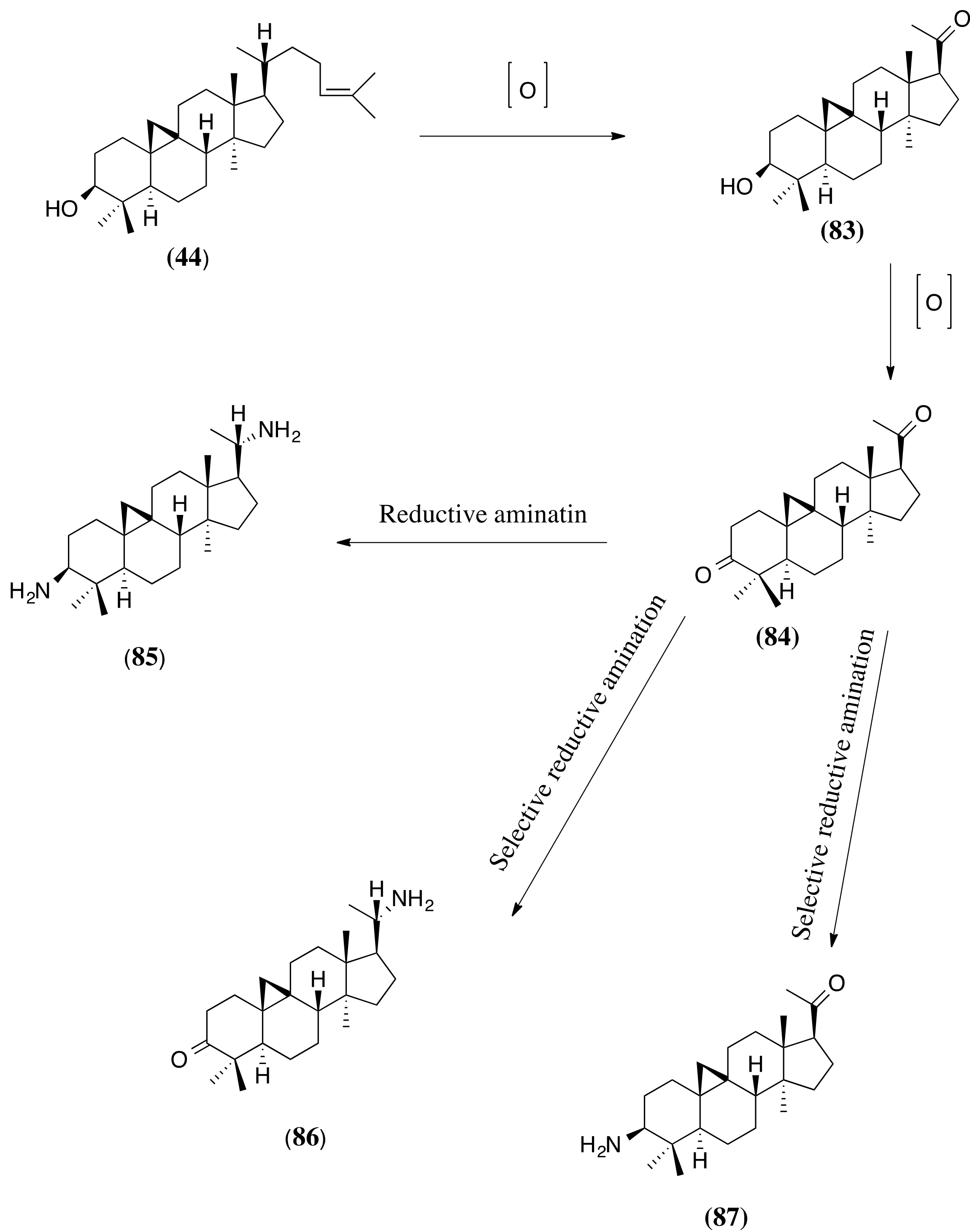
(82)

Table 1.2. Anti-AChE activity of *Buxus* alkaloids (**77**-**82**).

***Buxus* alkaloids**	**IC_{50} μM**
O^2-natafuranamine (**77**)	3.0
O^{10}-natafuranamine (**78**)	8.5
Buxafuranamide (**79**)	14
Spirofornabuxine (**80**)	6.3
Buxamine B (**81**)	7.5
Buxakashmiramine (**82**)	25.4

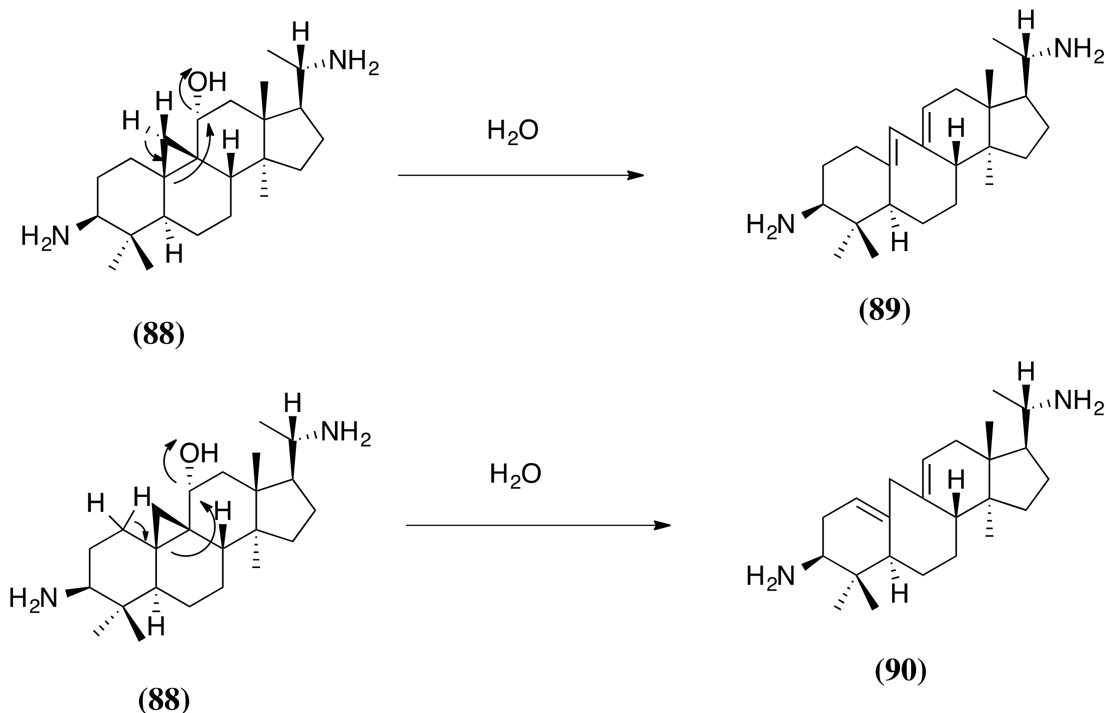
IC_{50} value indicates the concentration of compounds that inhibits the 50% activity of enzyme.

It is believed that *Buxus* alkaloids are derived from lanosterol (**43**), and cycloartenol (**44**). Both of these terpenoids are produced from squalene (**40**). Based on that the biosynthesis of **43** and **44** are summarized in **Scheme 1.2**. Cycloartenol **44** on oxidative cleavage of C-20 side chain may afford C-20 keto steroids (**83**) which may further undergo oxidation of C-3 hydroxyl group to give the 3,20 diketo steroids (**84**). Compound **84** may be the biogenetic precursors of *Buxus* alkaloids which on reductive amination may afford 3,20 diamino steroidal alkaloids (**85**). 20-Amino,3-keto steroidal alkaloids (**86**) or 3-amino,20-keto steroidal alkaloids (**87**) may be obtained by selective reductive amination of **84** (**Scheme 1.5**).[32]



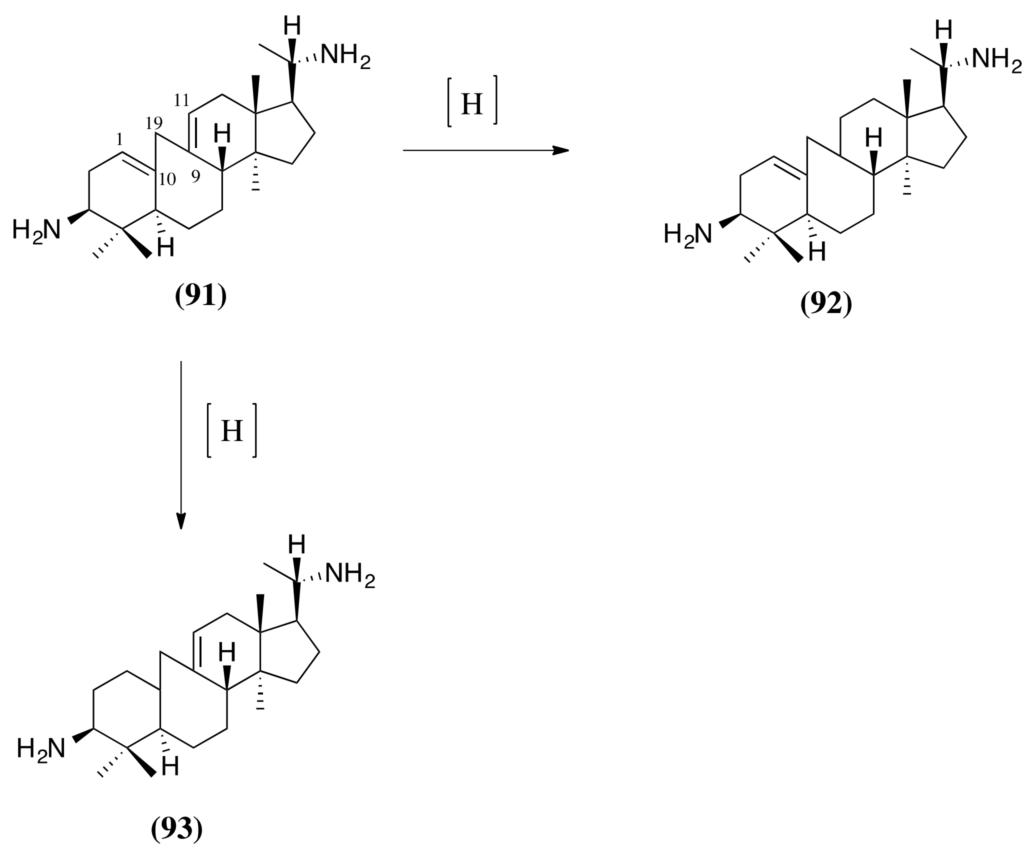
Scheme 1.5. Biosynthesis of *Buxus* alkaloids.

Buxus alkaloids of 9(10→19) series (**89**) and (**90**) might be produced in nature by the enzymatic ring opening of the cyclopropane (**88**), as summarized in **Scheme 1.6**.[32]

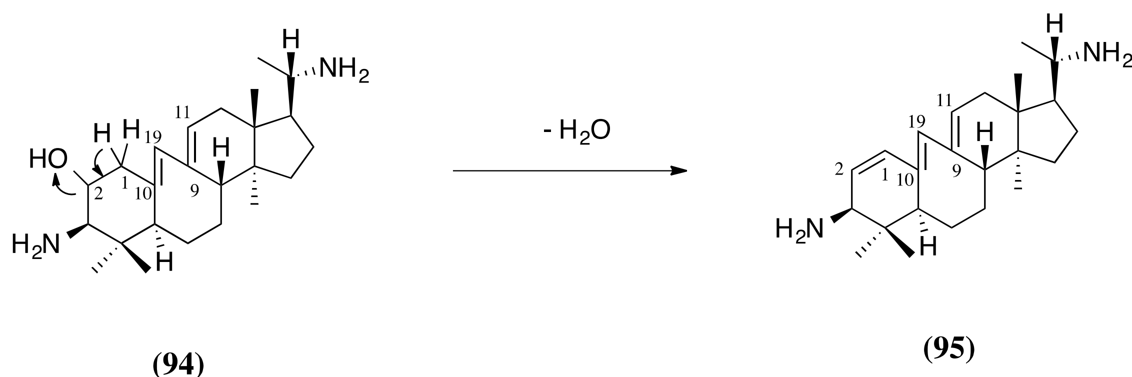


Scheme 1.6. Biosynthesis of *Buxus* alkaloids containing 9(10→19) *abeo* diene system.

Buxus alkaloids of 9(10→19) *abeo* $\Delta^{1\text{-}10}$ system (**92**), or 9(10→19) *abeo* $\Delta^{9\text{-}11}$ system (**93**) may arise in nature by the selective enzymatic reduction of the double bond $\Delta^{9\text{-}11}$ or $\Delta^{1\text{-}10}$ of the 9(10→19) *abeo* non conjugated alkaloids (**91**) as shown in **Scheme 1.7**. *Buxus* alkaloids containing 9(10→19) *abeo* triene system (**95**) may be produced by the β-elimination of a leaving group at C-2 of 9(10→19) *abeo* diene alkaloid (**94**) as shown in **Scheme 1.8**.[32]

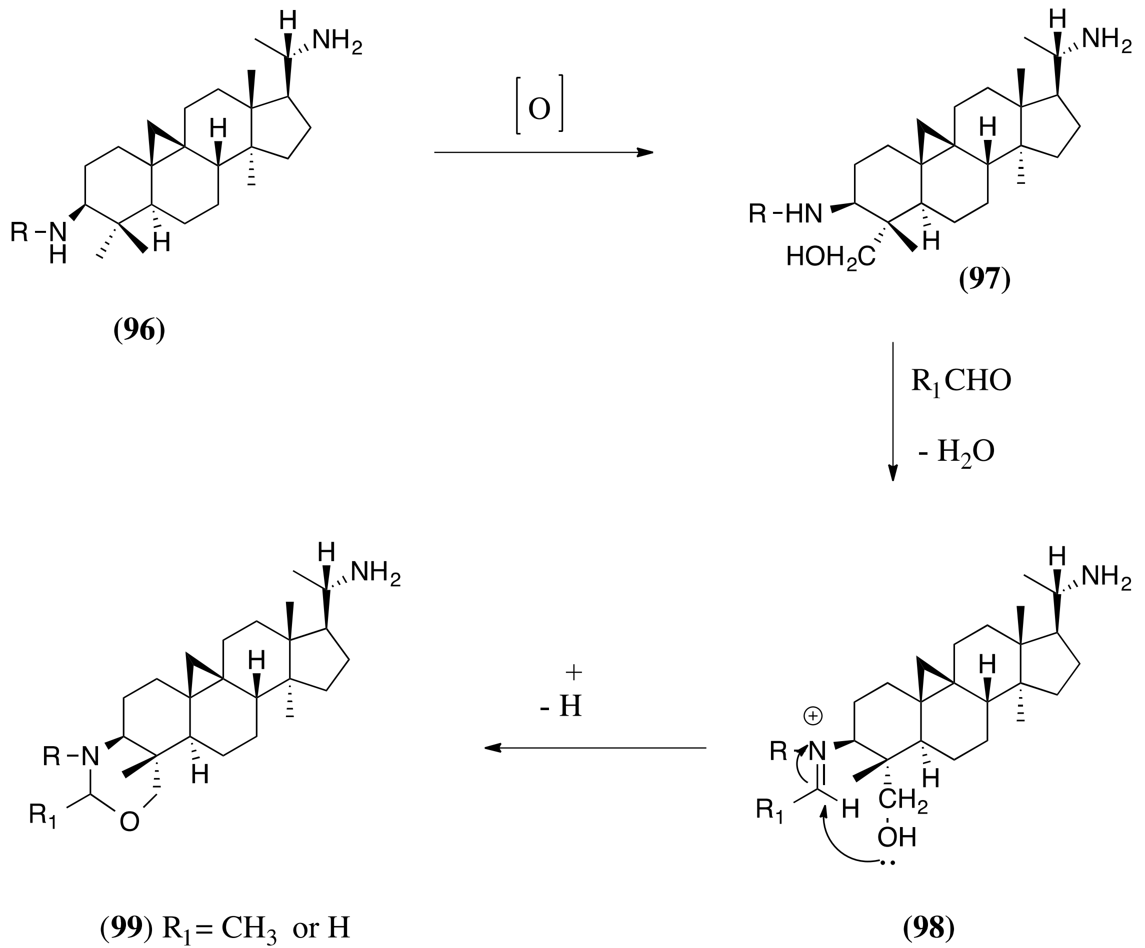


Scheme 1.7. Biosynthesis of *Buxus* alkaloids containing 9(10→19) *abeo* Δ^{1-10}, and *abeo* Δ^{9-11} system.

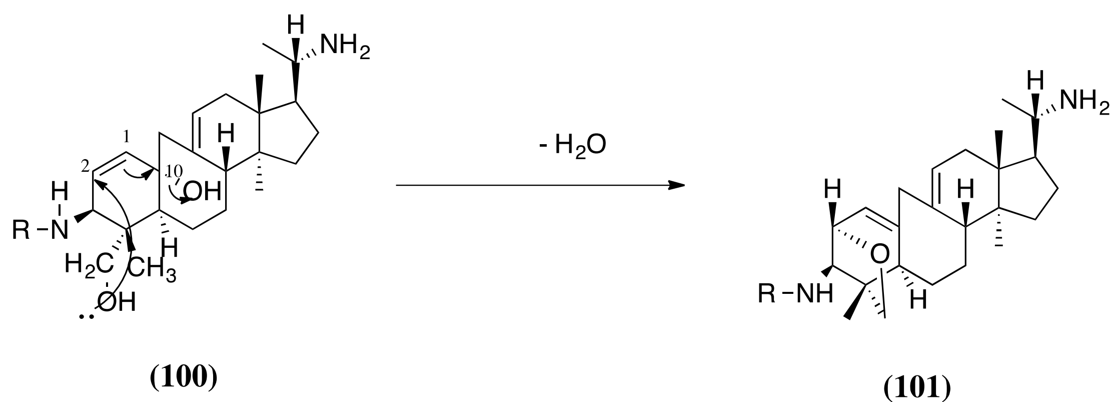


Scheme 1.8. Biosynthesis of *abeo* triene *Buxus* alkaloids.

Buxus alkaloids containing tetrahydrooxazine rings in their structure may be produced in nature by oxidation of C-31 methyl group of compound (**96**) to produce (**97**). The reaction of formaldehyde or acetaldehyde occurs on the C-3 amino group to produce ketimine (**98**). The hydroxyl group, which attached to C-31, attacks the ketimine to give tetrahydrooxazine rings (**99**). These rings also can be formed at C-16 and C-20 in case of C-20 amino-16-hydroxyl steroidal alkaloids (**Scheme 1.9**). Similarly, tetrahydrofuran rings may be biosynthesized from steroidal alkaloids (**100**) by the attack of C-31 hydroxyl group on the double bond at Δ^{1-2}, followed by rearrangement and the elimination of a good leaving group at the allylic position, resulting in steroidal alkaloids with a tetrahydrofuran ring (**101**) (**Scheme 1.10**).[32]



Scheme 1.9. Biosynthesis of tetrahydrooxazine ring in *Buxus* alkaloids.



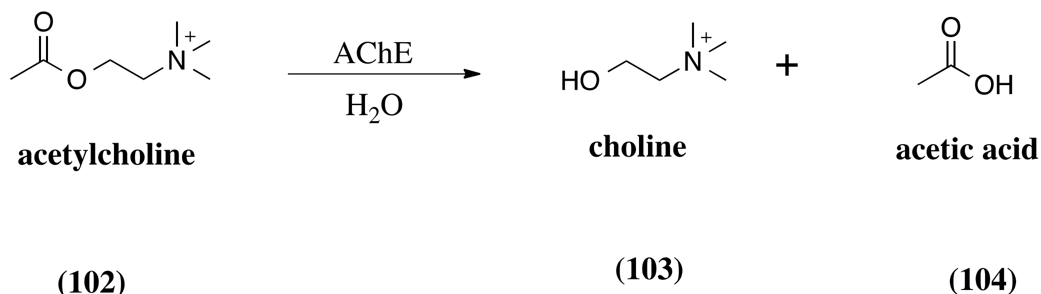
Scheme 1.10. Biosynthesis of tetrahydrofuran ring in *Buxus* alkaloids.

1.3 *BUXUS* ALKALOIDS AS ACETYLCHOLINESTERASE INHIBITORS

Alzheimer's disease (AD) was first discovered in 1901 by Alois Alzheimer, a German physician. It is the most common neurodegenerative disorder, accompanied by the loss of mental function, reduction of comprehension and social communication.[47] AD progresses in the brain via anatomical connections starting from the entorhinal cortex to all of the hippocampal subregions and cortical areas.[48,49] Nowadays, AD is affecting approximately 20 million people worldwide. Even though, the etiology of Alzheimer's disease is still not understood, scientists believe that it is due to various aspects such as life style, environmental conditions, and head injury accidents.[47,50]

There are different hypotheses regarding the pathogenesis of AD, of which amyloid and cholinergic are the most common. Majority of the recent histopathological studies show the present of amyloid plaques and neurofibrillary tangles in the brain of

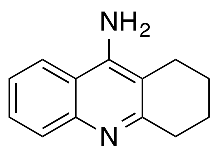
AD patients. Furthermore, the accumulation of β-amyloid (Aβ), a neurodegenerative protein, and an abnormal Tau protein production are observed in most cases of AD.[48] According to the cholinergic hypothesis, the deficiency of acetylcholine (ACh), an important neurotransmitter in the central nervous system (CNS), is the main cause of AD. This deficiency is a result of the excessive degradation of acetylcholine (**102**) to choline (**103**) and acetic acid (**104**) by acetylcholinesterase (AChE). Enhancing the cholinergic activity in the AD patients' brain by inhibiting AChE is one of the promising treatments of AD (**Scheme 1.11**).[51]



(102) **(103)** **(104)**

Scheme 1.11. Acetylcholinesterase catalysis of acetylcholine.

Currently, there are four FDA-approved acetylcholinesterase inhibitors used in the treatment of AD: tacrine (**105**), donepezil (**106**) rivastigmine (**107**), and galantamine (**108**). All of these drugs exhibit similar AChE inhibitory activity; however, they cause numerous side effects. For example, donepezil is the most commonly used drug to treat AD, while tacrine has been used very rarely due to its hepatotoxicity effect.[52,53] As a result, scientists are searching for compounds with higher AChE inhibitory activity and less side effects to use as a treatment for AD.



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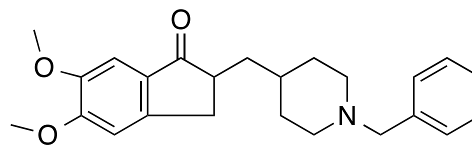

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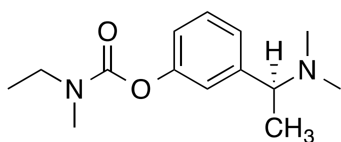

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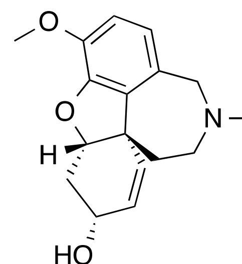
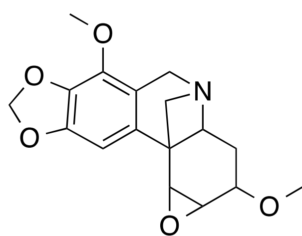


(108)

Alkaloids from various plants exhibit different levels of AChE inhibition activity, from moderate to strong. Some of these alkaloids include undulatine (**109**), taspine (**110**), and serpentine (**111**), which are isolated from *Nerine bowdenii*, *Magnolia x soulangiana*, and *Catharanthus roseue*, respectively.[54-56] Anti-AChE activity of representative alkaloids are shown in **Table 1.3**.

Table 1.3. AChE inhibitory activity of alkaloids (**109-111**).[54-56]

Alkaloid	Plant	IC_{50} (µM)
Undulatine **(109)**	Nerine bowdenii	37
Taspine **(110)**	Magnolia x soulangiana	0.3
serpentine (**111**)	Catharanthus roseue	0.7



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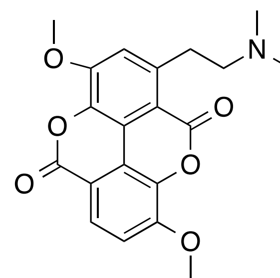

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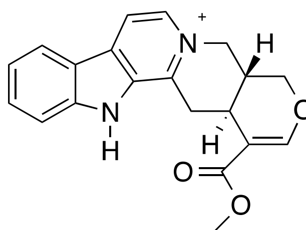

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CHAPTER 2

Isolation, Characterization, and Anti-AChE Activity of Steroidal Alkaloids from *Buxus macowanii*

2.1 INTRODUCTION

Genus *Buxus* is a rich source of steroidal alkaloids. The crude extracts of various species of this genus have been reported to treat rheumatism, malaria, and skin infections.[1] For example, the crude ethanolic extract of *B. sempervirens* exhibits anti-HIV activity and delays the progression of HIV-infected asymptomatic patients.[2] Phytochemical studies on various species of genus *Buxus* including *B. sempervirens, B. paplillosa, B. microphylla, B. hildebrandtii,* and *B. hyrcana*, have resulted in the isolation of over 200 new steroidal alkaloids.[1,2] A few of these alkaloids exhibit interesting biological properties. For instance, buxmicrophylline G exhibits cytotoxicity against HepG2 cells with an IC_{50} value of 0.89 μM.[3] These biological activities of *Buxus* alkaloids warrant further phytochemical investigation of these plants for the identification of natural products with new biomedical applications.

Genus *Buxus* is distributed in a wide ecological range of Europe, China, India, Caribbean, and Africa. Due to their small stature, no more than 3 meters tall, these plants are used as evergreen trees to decorate parks and gardens in Europe.[4] There are approximately 100 species of genus *Buxus,* 25 of these species have been thoroughly investigated while the remainder have not been chemically investigated.[4]

B. macowanii is a small evergreen tree commonly found in South Africa. Traditional healers use the water extract of this plant to enhance memory of elderly people. Based on the reported ethno-medical importance, this plant was collected from Umtamvuna Nature Reserve in South Africa. The methanolic extract was evaluated for AChE inhibitory activity and found to be active with an IC_{50} value of 30 µg/ml. The methanolic extract was defatted with hexane and it was discovered that hexane extract was inactive while defatted extract was active in this bioassay. Further fractionation of the bioactive extract was carried out using solvent-solvent extraction with dichloromethane at pH values 3.5, 7.0, and 9.5. The dichloromethane extracts obtained at pH 3.5, 7.0, and 9.5 were active in the bioassay with IC_{50} values of 50, 160, and 22 µg/ml, respectively. Check Wing Lam, M.Sc. student, University of Manitoba, and Andrew Wakeman, undergraduate student, University of Winnipeg, chemically investigated the dichloromethane extract obtained at pH 9.5. This thesis describes the chemical studies on dichloromethane extract (pH 3.5). A detailed column and thin layer chromatography (experimental section p. 64) afforded two novel steroidal alkaloids (**112**, and **113**) along with two known alkaloids (**114**, and **115**). This chapter describes the isolation and structure elucidation of compounds **112-115** with aid of UV, IR, mass, and 1D and 2D NMR spectroscopy. Additionally anti-AChE activity of compounds **112-115** is described in this chapter.

2.2 RESULTS AND DISCUSSION

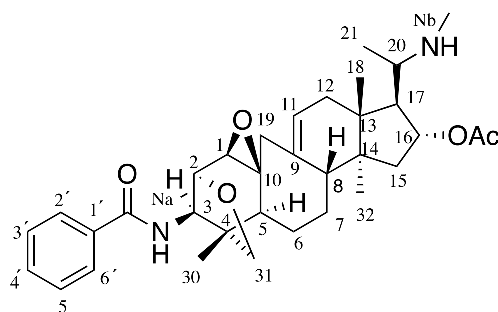
2.2.1 Structure elucidation of compounds (112-115)

2.2.1.1. N_b-Demethyl-6-deoxy-16-acetoxy O^2-natafuranamin (112)

Compound (**112**) was isolated as a colorless amorphous solid. The UV spectrum showed maximum absorption at 234 nm indicating the presence of benzamide chromophore.[5] The IR spectrum showed intense absorption bands at 1643 (α,β-unsaturated amide carbonyl); 1711 (ester carbonyl), and 1089 (C-O) cm^{-1}. The High-Resolution Electron-Impact Mass Spectrum (HREIMS), along with ^{1}H- and ^{13}C-NMR spectroscopic data provided the molecular formula, $C_{34}H_{46}N_2O_5$ [M^+ *m/z* 562.3402 (observed), 562.3407 (calcd)] indicating thirteen degrees of unsaturation. The base peak at *m/z* 58 was due to the cleavage of the ring D side chain containing N_b-methyl amino group at C-20.[1] Another intense ion at *m/z* 105 was due to the loss of a benzoyl group.[2]

The ^{1}H-NMR spectrum ($CDCl_3$, 400MHz) of **112** showed the resonance of three three-proton singlets at δ 0.83, 0.97, and 1.16 due to the protons of C-18, C-32, and C-30 tertiary methyl groups, respectively. These methyl groups were substituted at C-13, C-14, and C-4 quaternary carbons, respectively. A three-proton doublet centered at δ 1.26 was assigned to the C-21 secondary methyl protons. Another three-protons singlet resonated at δ 1.96 was ascribed to the proton of acetyl methyl group. A broad singlet at δ 2.77, integrating for three protons, was due to N_b-CH_3 group. A two-proton singlet at δ 3.89 was assigned to the C-31 methylene protons. Its downfield chemical shift value indicated the presence of a geminal oxygen functionality. The C-31 methyl group was absent in

the ^{1}H-NMR spectrum and this methyl is reported to undergo oxidation in *Buxus* alkaloids.[6,7] It has been reported in the literature that C-4-hydroxy methylene protons of *Buxus* alkaloids resonate as AB doublets in the range of δ 3.70-4.00. This two-proton singlet resonated at δ 3.89 and exhibited ^{1}H/^{13}C one-bond shift correlation with C-31 (δ 83.3). This carbon was methylene as indicated by the ^{13}C DEPT spectrum. This signal was assumed to be due to the C-31 methylene protons. In order to resolve the ^{1}H-NMR singlet (δ 3.89) into AB doublet, the ^{1}H-NMR spectrum of **112** was recorded in acetone-d_6. This ^{1}H-NMR spectrum showed the resonance of this signal as AB doublet at δ 3.85 and 3.98 (J = 8.9 Hz). These AB doublets showed connectivity with C-31. These NMR spectroscopic data confirmed the signal at δ 3.89 was due to the C-31 methylene protons. The C-2 methine proton resonated at δ 4.33, while C-3 methine proton appeared at δ 4.53. Two multiplets at δ 5.27 and 5.23, integrating for one proton each, were assigned to C-11 and C-16 methine protons, respectively. An amide NH proton resonated as doublet at δ 7.66 while aromatic protons resonated at δ 7.52, 7.59, and 7.79.



The ^{13}C-NMR (APT) ($CDCl_3$, 100 MHz) showed the resonance of 34 carbons. A combination of APT and DEPT 135 spectra showed the presence of six methyl, six methylene, fourteen methine, and eight quaternary carbons in compound **112**. The HSQC

spectrum was also recorded in order to determine the $^{1}H/^{13}C$ one-bond connectivity of protonated carbons. The ^{1}H, ^{13}C NMR chemical shift assignments, and HSQC spectroscopic data of **112** are shown in **Table 2.1.**

The COSY-45° and TOCSY spectra of **112** revealed the presence of four-different spin systems “**a-d**” in the molecule. The first spin system “**a**” was due to the phenyl moiety. The C-2´/C-6´ protons (δ 7.79) showed cross peaks with C-3´/C-5´ protons (δ 7.52). The later protons exhibited cross peaks with the para C-4´ proton (δ 7.59). The second spin system “**b**” was composed of fragment of coupled protons of H-1 to amide NH. The COSY-45° spectrum showed the vicinal couplings of C-1 methine proton (δ 3.29) with C-2 methine proton (δ 4.31). The later exhibited cross peaks with C-3 methine proton (δ 4.51), which in turn showed vicinal coupling with NH (δ 7.67). The third spin system “**c**” was traced from the vicinal couplings of C-11 methine proton (δ 5.28) with C-12 methylene protons (δ 1.82, and 2.13). The former exhibited allylic couplings with C-8 methine proton (δ 2.39), which in turn showed cross peaks with the C-7 methylene protons (δ 2.05, and 1.75). H_2-7 showed cross peaks with the C-6 methylene protons (δ 1.34). The later exhibited vicinal couplings with the C-5 methine proton (δ 3.32). Allylic couplings of the C-19 methylene protons (δ 2.80, and 2.31) with the H-11 were also observed in the TOCSY spectrum. The fourth spin system “**d**” was composed of coupled proton of ring **D** and its side chain. It was traced form the vicinal coupling of C-16 (δ 5.23) methine proton with C-15 methylene (δ 1.5, and 2.05), and C-17 (δ 2.31) methine protons. H-17 in turn showed ^{1}H-^{1}H spin correlation with H-20 (δ 2.01) which further showed cross peaks with C-21 methyl protons (δ 1.26). These partial structures “**a-d**” are

shown in **Figure 2.1**.

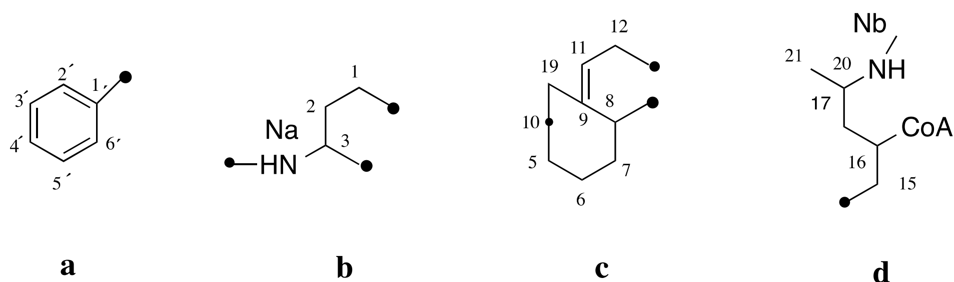


Figure 2.1. Partial structure of **112** by COSY-45° spectrum.

The HMBC spectrum was recorded to determine the chemical shift of quaternary carbons and to build structure **112** by connecting partial structure "**a-d**" The H-2´/ H-6´ (δ 7.79) of fragment "**a**" exhibited correlation with C-1´ (δ 134.1) and the amide carbonyl carbon (δ 167.2). The amide proton showed a long-range hetronuclear coupling with the carbonyl carbon. These HMBC data helped to connect partial structure "**a**" with partial structure "**b**" through an amide bond. H-3 (δ 4.51) showed cross peaks with the quaternary C-4 (δ 44.4), and C-5 (δ 65.3) of partial structure "**c**". H-5 (δ 3.32) exhibited cross peaks with C-3 (δ 56.7), C-4 (δ 44.4), and C-10 (δ 58.8). H_3-30 showed cross peaks with C-3, and C-31 (δ 83.3). These HMBC observations indicated the linkage of C-3 with C-5 through quaternary carbon C-4 that was bonded with C-30 methyl, and C-31 methylene groups. H_2-31 (δ 3.89) showed cross peaks with C-2 (δ 72.9), C-3 and C-4, and C-30 (δ 21.4) indicating the connection of C-31 with C-2 via an ether linkage. The C-2 showed connection with C-1 (δ 63.8), and C-3. H_2-19 (δ 2.31, 2.80) exhibited cross peaks with C-8 (δ 41.9), C-9 (δ 135.1), C-10 (δ 58.8), C-5 (δ 65.3), and C-11 (δ 121.2). Based on these HMBC observations, C-19 was connected with C-10 and C-5. Similarly other HMBC observations led us to establish gross structure **112** for this new alkaloid.

Important HMBC correlations of **112** are shown in **Figure 2.2.**

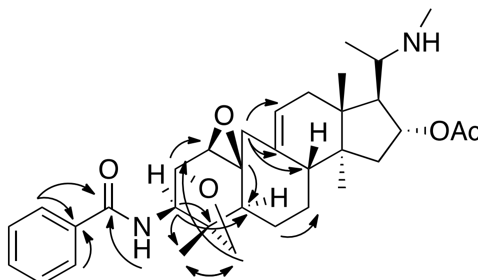


Figure 2.2. Important HMBC correlations of **112**.

Stereochemistry at all chiral centers was established with the aid of NOESY spectrum. It has been reported in the literature that H-3, H-5, H-17, and H_3-32 are α-oriented in this class of natural products, while H-8, H_3-18, and H_3-30 are β-oriented in *Buxus* alkaloids.[7,8] This was confirmed from the NOESY spectrum in which H-3 (δ 4.51) showed an NOE with H-5 (δ 3.32). The later showed cross peaks with H_3-32 (δ 0.97). These NOSEY data suggested a *cis* relationship among these groups and based on these observations α-stereochemistry was proposed for H-3, H-5, H-17, and H_3-32. Similarly, the NOSEY spectrum indicated a *cis* relationship among H-2, H-8, H_3-18, H_3-30, and H-16 and hence β-orientation was assumed for these groups. The H-1 (δ 3.29) showed an NOE with H-3, and H-5 suggesting the α-orientation of H-1. These spectroscopic studies led us to assign structure **112** for this new steroidal alkaloid and named as N_b-demethyl-6-deoxy-16-acetoxy O^2-natafuranamin.

Table 2.1. H^1 and ^{13}C NMR spectroscopic data (400 and 100 MHz) of N_b-demethyl-6-deoxy-16-acetoxy O^2-natafuranamin (**112**) in $CDCl_3$.

Position	δ_C	**Multiplicity†**	δ_H
1	63.8	CH	3.29, d
2	72.9	CH	4.31, m
3	56.7	CH	4.51, m
4	44.4	C	-
5	65.3	CH	3.32
6	29.2	CH2	1.34, m
7	36.7	CH2	1.75, m, 2.05, m
8	41.9	CH	2.39, m
9	135.1	C	-
10	58.8	C	-
11	121.2	CH	5.25,m
12	36.8	CH2	2.13, m, 1.82, m
13	45.1	C	-
14	47.1	C	-
15	43.4	CH2	2.05, m, 1.50, m
16	74.7	CH	5.23,m
17	55.3	CH	2.31, m
18	15.8	CH3	0.83, s
19	42.5	CH2	2.31, d, 2.80, d
20	55.9	CH	2. 08, m
21	11.6	CH3	1.26, d
30	21.4	CH3	1.16, s
31	83.3	CH2	3.89, s
32	17.1	CH3	0.97, s
OCNH	167.2	C	1.96, s
OCNH			7.67, d
NCH_3	44.4	CH3	2.77, s
$OCOCH_3$	169.7	C	-
$OCOCH_3$	21.3	CH3	1.96, s
1´	134.1	C	-
2´	126.5	CH	7.79, d
3´	128.5	CH	7.52, m
4´	131.6	CH	7.59, m
5´	128.5	CH	7.52, m
6´	126.5	CH	7.79, d

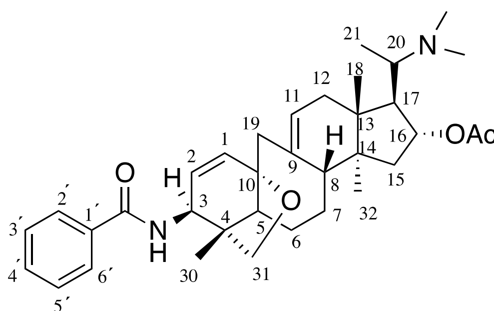
†Multiplicity was determined by APT and DEPT spectra

2.2.1.2 6-Deoxy-16-acetoxy O^{10}-natafuranamin (113)

Compound (**113**) was isolated as white amorphous powder. Its UV spectrum showed the absorption maximum at 224 nm indicating the presence of benzamide chromophore.[5] The IR spectrum displayed intense absorption bands at 1645 (α,*β*-unsaturated amide carbonyl); 1709 (ester carbonyl), and 1095 (C-O) cm^{-1}. The HREIMS of **113** showed the molecular ion peak at *m/z* 560.3610 that provided molecular formula $C_{35}H_{48}N_2O_4$ (560.3614, calcd) indicating thirteen degrees of unsaturation. The base peak at *m/z* 72 was indicative of the presence of *N*,*N*-dimethyl group at C-20.[2] Another intense ion at *m/z* 105 was due to the loss of a benzoyl group.[2]

The ^{1}H-NMR spectrum (acetone-d_6, 400MHz) of compound **113** showed the resonance of three three-proton singlets at δ 0.83, 0.95, and 1.17 due to the protons of three tertiary methyl groups (C-18, C-32 and C-30) substituted at C-13, C-14, and C-4 quaternary carbons, respectively. A three-proton doublet centered at δ 1.40 was assigned to the C-21 secondary methyl protons. A one-proton multiplet resonated at δ 1.89 due to the C-20 methine proton. A three-proton singlet resonated at δ 1.96 was assigned to the protons acetyl methyl group. A six-proton broad singlet appeared at δ 2.80 was due to *N*-$(CH_3)_2$ group. Two AB doublets, integrating for one proton each, appeared at δ 3.59, and δ 3.62 (J = 8.4 Hz) were assigned to the C-31 methylene protons. A downfield one-proton multiplet appeared at δ 4.78 was ascribed to C-3 methine proton. Another one-proton multiplet resonated at δ 5.12 was due to C-16 methine proton. Its downfield chemical shift indicated the presence of a geminal acetoxy group. A broad singlet, integrated for one proton, resonated at δ 5.51 was assigned to C-11 olefinic proton. A

multiplet, integrated for two protons, appeared at δ 5.89 were assigned to C-1 and C-2 olefinic protons. A doublet, integrated for one proton, resonated at δ 6.99 was due to an amidic NH. A two-proton multiplet at δ 7.48 was assigned to chemically equivalent C-3´ and C-5´ aromatic protons. A one-proton multiplet at δ 7.55 was due to C-4´ aromatic proton. A two-proton doublet centered at δ 7.87 was assigned to chemically equivalent C-2´ and C-6´ aromatic protons.



A combination of APT ^{13}C-NMR (aceton-d_6, 100 MHz) and DEPT 135 spectra of compound **113** revealed the presence of seven methyl, six methylene, fourteen methine, and eight quaternary carbons. The HSQC spectrum of **113** was used to establish the ^{1}H/^{13}C one-bond correlation of all protonated carbons. The ^{1}H- and ^{13}C-NMR chemical shift assignments, and HSQC data of **113** are presented in **Table 2.2**.

The COSY-45° and TOCSY spectra of **113** showed the presence of five-isolated spin system "**a-e**" in the molecule. The first spin system "**a**" was due to the phenyl moiety. H-2´/H-6´ (δ 7.87) showed cross peaks with H-3´/H-5´ (δ 7.48). The later exhibited cross peaks with H-4´ (δ 7.55). The second spin system "**b**" began with an amide proton (δ 6.99), which exhibited cross peaks with the C-3 methine proton (δ 4.78).

The later showed cross peaks with H-1/H-2 (δ 5.89). The third spin system "**c**" started with C-5 methine proton (δ 3.61) that showed vicinal couplings with the C-6 methylene protons (δ 1.97, and 2.21). The later exhibited cross peaks with H_2-7 (δ 1.40) which further showed coupling with the C-8 methine proton (δ 2.92). The fourth spin system "**d**" showed the couplings between C-11 olefinic proton at δ 5.50 and C-12 methylene protons (δ 1.97, and 2.21). Also, allylic couplings of C-19 methylene protons (δ 2.69, and 2.79) with H-11 were also observed in the COSY and TOCSY spectra. The last spin system "**e**" showed the coupling between C-15 methylene proton (δ 1.21, 1.63), and C-16 methine proton δ 5.12. The later exhibited coupling with C-17 methine proton (δ 2.20) that in turn showed vicinal coupling with C-20 methine proton at δ 1.89, which further exhibited cross peaks with C-21 methyl protons at δ 1.40. These partial structures "**a-e**" are shown in **Figure 2.3.**

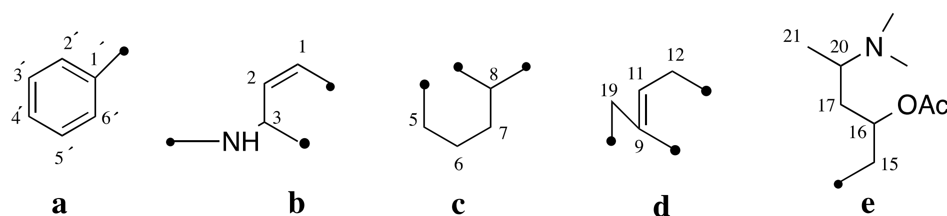


Figure 2.3. Spin systems of **113** determined by COSY-45° spectrum.

The HMBC spectrum of **113** showed the long-range couplings of H-2´/H-6´ (δ 7.87) of partial structure "**a**" with C-1´ (δ 135.4) and amide carbonyl carbon (δ 166.3). H-2 at (δ 5.89) showed cross peaks with the C-4 (δ 47.7), C-30 (δ 21.2), and C-31 (δ 79.7). H-5 (δ 3.61) showed cross peaks with C-2 (δ131.1), C-19 (δ 44.1), and C-30. These HMBC observations led us to connect partial structures "**a**" and "**b**" through an amide

bond and partial structure “**b**” and “**c**” through quaternary carbon C-4 and C-10 (δ 80.8). Similarly partial structure “**c**”, “**d**”, and “**e**” were connected to build the steroidal skeleton and to satisfy the thirteen degrees of unsaturation. Important HMBC interactions of **113** are shown in **Figure 2.4**.

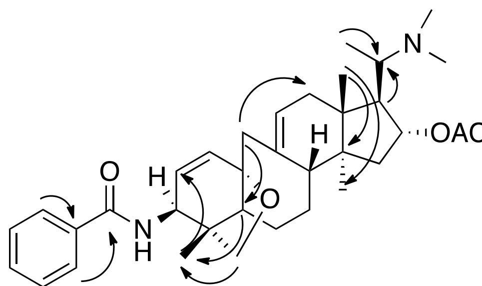


Figure 2.4. Important HMBC correlations of **113**.

The NOSEY spectrum of **113** revealed the same stereochemistry at all chiral centers (C-3, C-4, C-5, C-8, C-13, C-14, C-17, and C-20) as those of previously discussed compound **112**. H-16 (δ 5.23) showed cross peaks with H_3-18 (δ 0.83) and H-8 (δ 2.39) indicating its β-stereochemistry. These spectroscopic studies led us to establish structure **113** for this new natural product and named as 6-deoxy-16-acetoxy O^{10}-natafuranamin.

Table 2.2. ^{1}H and ^{13}C NMR spectroscopic data (400 and 100 MHz) for 6-deoxy-16-acetoxy O^{10}-natafuranamin (**113**) in acetone-d_6.

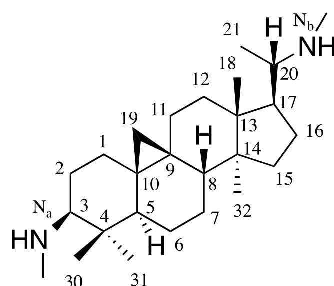
Position	δ_C	Multiplicity†	δ_H
1	134.1	CH	5.89, d
2	131.1	CH	5.89, m
3	53.8	CH	4.78, m
4	47.7	C	-
5	66.3	CH	3.61, m
6	38.5	CH_2	1.97, m, 2.21, m
7	44.9	CH_2	1.40, m
8	43.1	CH	2.92, m
9	135.4	C	-
10	80.8	C	-
11	125.5	CH	5.50, br s
12	38.5	CH_2	1.97, m, 2.21, m
13	46.9	C	-
14	49.1	C	-
15	35.4	CH_2	1.21, m, 1.63, m
16	76.8	CH	5.12, m
17	57.7	CH	2.20, m
18	17.6	CH_3	0.83, m
19	44.1	CH_2	2.69, m, 2.79, m
20	58.5	CH	1.89, m
21	12.7	CH_3	1.40, d
30	21.2	CH_3	1.17, s
31	79.7	CH_2	3.59, d, 3.62, d
32	18.3	CH_3	0.95, s
OCNH	166.3	C	
OCNH			6.99, d
N $(CH_3)_2$	43.1	CH_3	2.80, s
$OCOCH_3$	170.1	C	-
$OCOCH_3$	22.2	CH_3	1.96
1´	135.4	C	-
2´	128.9	CH	7.87, d
3´	129.7	CH	7.48, m
4´	132.9	CH	7.55, m
5´	129.8	CH	7.48, m
6´	128.9	CH	7.87, d

†Multiplicity was determined by APT and DEPT spectra

2.2.1.3 Cycloprotobuxine-D (114)

Compound (**114**) was isolated as white amorphous powder. Its UV spectrum showed terminal absorption indicated the lack of conjugated system. The IR spectrum displayed intense absorption band at 3399 (NH) cm^{-1}. The Electron-Impact Mass Spectrum (EIMS) of **114** showed the molecular ion peak at *m/z* 386. A combination of EIMS, ^{1}H- and ^{13}C-NMR spectroscopic data provided its molecular formula, $C_{26}H_{46}N_2$. The base peak at *m/z* 58 was due to the cleavage of the ring D side chain containing *N*-methyl amino group at C-20.[1] The base peak at *m/z* 57 was due to the cleavage of ring a to *N*-methyl amino group.[1]

The ^{1}H-NMR spectrum ($CDCl_3$, 400MHz) of **114** showed the resonance of AB doublets, integrating for one proton each at δ 0.41, and 0.57 due to C-19 cyclopropyl methylene protons. Four three-proton singlets resonated at δ 0.94, 1.01, 1.01, and 1.17 due to the protons of the tertiary C-30, C-31, C-32, and, C-18 methyl groups. These methyl groups were substituted at C-4, C-14, and C-13 quaternary carbons, respectively. A three-proton doublet centered at δ 1.31 was assigned to the C-21 secondary methyl group. A two three-proton singlets resonated at δ 2.88 and 2.68 were assigned to N_a and N_b-methyl protons, respectively. A one-proton appeared as multiplet at δ 2.98 was due to the C-20 methine proton. A one-proton multiplet appeared at δ 3.22 was assigned to the C-3 methine proton.



The ^{13}C APT and DEPT 135 spectra of **114** revealed the presence of seven methyl, nine methylene, five methine, and five quaternary carbons. The HSQC spectrum of **114** was used to assign the $^{1}H/^{13}C$ one-bond shift correlation of all protonated carbons. The ^{1}H- and ^{13}C-NMR chemical shift values, and HSQC data of **114** are shown in **Table 2.3**. These spectroscopic studies led us to characterize compound **114** as a known steroidal alkaloid, cycloprotobuxine-D. The UV, IR, mass, and NMR spectroscopic data of **114** was similar to these of cycloprotobuxine-D, reported in the literature.[9] Previously, compound **114** was purified from *Buxus. balearica, B. harlandi, and B. sempervirens*.[10,11] This is the first time, we have isolated this compound from *B. macowanii*.

Table 2.3. ^{1}H and ^{13}C NMR spectroscopic data (400 and 100 MHz) for cycloprotobuxine-D (**114**) in $CHCl_3$.

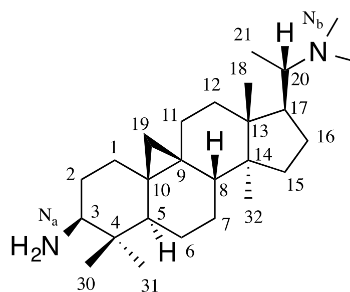
Position	δ_C	Multiplicity†	δ_H
1	26.3	CH_2	1.99, m, 2.19, m
2	26.8	CH_2	1.98, m, 2.21, m
3	65.5	CH	3.25, m
4	38.4	C	-
5	47.3	CH	1.42, m
6	32.4	CH_2	1.21, m, 1.66, m
7	26.8	CH_2	1.32, m, 1.51, m
8	47.3	CH	1.50, m
9	19.8	C	-
10	19.8	C	-
11	20.7	CH_2	0.77,m, 1.70, m
12	25.3	CH_2	0.85, m, 1.43, m
13	45.7	C	-
14	48.4	C	-
15	35.4	CH_2	1.35, m, 1.51, m
16	31.4	CH_2	1.30, m, 1.59, m
17	48.8	CH	2.09, m
18	19.0	CH_3	0.93, s
19	28.8	CH_2	0.41, d, 0.57, d
20	60.4	CH	2.98, m
21	11.7	CH_3	1.31, d
30	26.4	CH_3	1.17, s
31	14.7	CH_3	1.02, s
32	18.2	CH_3	1.01, s
N_a (CH_3)	35.4*	CH_3	2.68
N_b (CH_3)	43.2*	CH_3	2.88

†Multiplicity was determined by APT and DEPT spectra. *Assignments are interchangeable

2.2.1.4 Cycloprotobuxine-F (115)

Compound (**115**) was isolated as yellow colored gum. Its UV spectrum showed terminal absorption indicated the lack of conjugated system. The IR spectrum displayed intense absorption band at 3411 (NH) cm^{-1}. The EIMS of **115** showed the molecular ion peak at *m/z* 386. The molecular formula, $C_{26}H_{46}N_2$ was determined using a combination of EIMS, H- and ^{13}C-NMR spectroscopic data. The base peak at *m/z* 72 was inductive of the present of *N*,*N*-dimethyl group at C-20.[2] Another base peak at *m/z* 43 was due to the cleavage of ring **A** to amino group.

The ^{1}H-NMR spectrum (pyridine-d_5, 400MHz) of **115** showed the resonance of AB doublets, integrating for one proton each, at δ 0.24, and 0.47 due to C-19 cyclopropyl methylene protons. Four three-proton singlets resonated at δ 1.36, 1.21, 0.98, and 0.93 due to the protons of the tertiary C-30, C-31, C-32 and, C-18 methyl groups. These methyl groups were substituted at C-4, C-14, and C-13 quaternary carbons, respectively. A three-proton doublet centered at δ 0.90 was assigned to the C-21 secondary methyl group. A one-proton appeared as multiplet at δ 1.93 was due to the C-20 methine proton. A six-proton singlet resonated at δ 2.29 was assigned to the N_b-dimethyl protons. A one-proton multiplet appeared at δ 3.25 was assigned to the C-3 methine proton.



The ^{13}C APT and DEPT spectra of **115** revealed the presence of seven methyl, nine methylene, five methine, and five quaternary carbons. The HSQC spectrum of **115** was used to assign the ^{1}H/^{13}C one-bond correlation of all protonated carbons. The ^{1}H, ^{13}C NMR chemical shift values, and HSQC data of **115** are shown in **Table 2.4**. These spectroscopic studies led us to characterize compound **115** as a known steroidal alkaloid, cycloprotobuxine-F. The UV, IR, mass, and NMR spectroscopic data of **115** was similar to these of cycloprotobuxine-F, reported in the literature.[12] Previously, compound **115** was purified from *Buxus. madagascarica*.[13] This is the first time, we have isolated this compound from *B. macowanii*.

Table 2.4. ^{1}H and ^{13}C NMR spectroscopic data (400 and 100 MHz), for cycloprotobuxine-F (**115**) in pyridine-d_5.

Position	δ_C	**Multiplicity†**	δ_H
1	28.8	CH2	1.98, m, 2.15,m
2	29.1	CH2	2.45, m, 2.03, m
3	3.3	CH	3.25,m
4	50.2	C	-
5	52.2	CH	2.01, m
6	27.1	CH2	1.24, m, 0.98, m
7	36.6	CH2	1.39, m, 1.01, m
8	48.8	CH	1.37
9	22.2	C	-
10	22.2	C	-
11	22.5	CH2	1.61, m, 0.7, m
12	27.7	CH2	1.82, m
13	40.2	C	-
14	45.7	C	-
15	33.2	CH2	1.27, m, 1.55, m
16	33.8	CH2	1.63, m, 1.35, m
17	48.8	CH	1.52, m
18	19.9	CH3	0.95, s
19	30.4	CH2	0.25, d, 0.49, d
20	52.2	CH	1.95, m
21	11.1	CH3	0.90, d
30	27.5	CH3	1.36, s
31	16.6	CH3	1.21, s
32	20.4	CH3	0.92, s
$N_b(CH_3)_2$	40.9	CH3	2.29, s

†Multiplicity was determined by APT and DEPT spectra

2.2.1.5 Acetylcholinesterase Inhibitory Activity of 112-115

Compounds **112-115** were evaluated for AChE inhibitory activity and showed various levels of Anti-AChE activity. Compound **112** exhibited significantly higher AChE inhibitory activity (IC_{50} = 4.7 µM), compared to the rest of the compounds. The higher potency of **112** might be due to the presence of the epoxy functionality at C-1 and C-10, and the tetrahydrofuran ring. Compound **113** exhibited enhanced AChE inhibitory activity, with an IC_{50} value of 11.0 µM, compared to compounds **114** and **115.** The higher potency of **113** might be due to the presence of a tetrahydrofuran ring incorporated in its structure. These results are consistent with the previously reported AChE inhibitory activity of O^2-natafuranimine and other *Buxus* alkaloids, suggesting that higher potency of **112** and **113** are due to the presence of tetrahydrofuran ring incorporated in their structures.[14,15] The acetylcholinesterase inhibitory activity of compounds **112-115** is shown in **Table 2.5**.

Table 2.5. Acetylcholinesterase inhibitory activity of compounds **112-115**

Compounds	AChE IC_{50} (µM)
N_b-Demethyl-6-deoxy-16-acetoxy O^2-natafuranamin (**112**)	4.7±0.5
6-Deoxy-16-acetoxy O^{10}-natafuranamin **(113)**	11.0±0.7
Cycloprotobuxine-D (**114)**	100±2.0
Cycloprotobuxine-F **(115)**	120±2.2
Huperzine	0.53±0.5

2.3 EXPERIMENTAL

2.3.1 GENERAL

The silica gel 60 Å (40-63 microns) for column chromatography was purchased from Caledon Laboratory Chemicals, Canada. Thin layer chromatography was performed on EMD silica gel 60 F_{254} pre-coated sheets that were also purchased from Caledon Laboratory Chemicals, Canada. The UV spectra were acquired using a Shimadzu UV-2501 PC spectrophotometer. IR spectra were recorded on a Varian 1000 FT-IR (Scimitar Series) in methanol or chloroform. The 1D-NMR (^{1}H, APT, and DEPT) and 2D-NMR (COSY-45°, TOCSY, HSQC, HMBC, and NOESY) were acquired on a Bruker Avance 3 400 MHz spectrometer. All of the above mentioned NMR experiments were carried out using deuterated solvents including $CDCl_3$, pyridine-d_5, and acetone-d_6. All of these solvents were purchased from CDN Isotopes, Canada. All of solvents, used during the fractionation of crude extract, isolation, and purification of compound 112-115 were purchased from Cledon Laboratory Chemicals. Diethylamine, used as one of the mobile phase for TLC was purchased from EMD, Canada. Bismuth (III) nitrate, used to prepare dragendroff's reagent to visualize alkaloids was purchased from Alfa Aesar. The TLCs plates were visualize using a Handheld UV-lamp (UVGL-58 254/365 nm).

2.3.2 PLANTS MATERIAL

The bark of *Buxus macawanni* was collected from Umtamvuna Nature Reserve in South Africa in collaboration with Robert Gengan, Department of Chemistry, Durban University, South Africa during September 2007. The plant was identified by Mkhipheni

A Ngwenya, Scientific Officer, South Africa National Biodiversity Institute, South Africa. A voucher specimen (NH132295-0) was deposited in the South Africa National Biodiversity Institute, Durban, South Africa.

2.3.3 COMPOUNDS EXTRACTION AND ISOLATIONS

Bark of *B. macowanii* (9.6 Kg) was dried and ground into powder. The powdered plant material was extracted with methanol at room temperature. The extraction process was carried out by Mr. Abin James, a Ph.D student, Durban University of Technology, South Africa. The methanolic extract was rotavaped to prepare gum (1.4 Kg). This gum was shipped to a natural product chemistry lab, University of Winnipeg. Ms. Cheuck Wing Lam, M.Sc. student, University of Manitoba, carried out solvent-solvent extraction using dichloromethane at pH 3.5, 7.0, and 9.5. All of these extracts were evaluated for anti-AChE activity and found to be active with IC_{50} values of 50, 160, and 22 µg/ml, respectively. The dichloromethane extract (pH 9.5) was phytochemically studied by Ms. Lam, and Mr. Andrew Wakeman. The phytochemical investigation of dichloromethane extract, obtained at pH 3.5 was carried out and a detailed isolation procedure is summarized as follows.

The dichloromethane extract obtained at pH 3.5 (100 g) was loaded onto a silica gel column (primary column) and the column was eluted with hexane-dichloromethane (0%-100%) and dichloromethane-methanol (0%-100%) to afford 456 fractions. These fractions were pooled on the basis of analytical TLCs to yield 98 fractions.

Fraction BMPF 184-230 (10.2 g), obtained from the primary column, was again

loaded onto a silica gel column. This column was eluted with hexane-chloroform (0%-100%) and chloroform–methanol (0%-100%). It again provided 368 fractions, which were combined on the basis of similar R_f values on analytical TLCs to afford 32 fractions.

Fraction BMSF 148 (2.3 g), obtained on elution of the secondary column with 40% hexane and 60% chloroform, was again loaded onto a silica gel column. This column was eluted using hexane-chloroform (0%-100%), and chloroform-methanol (0%-100%) as a mobile phase. This mobile phase contained 1 ml of diethylamine to provide a fraction BMTF 166 (35.0 mg), which showed the presence of one major compound along with several minor compounds. It was decided to isolate major compound, to be present in enough quantity for characterization. Preparative TLC of this fraction was carried out using hexane-chloroform-methanol-diethylamine (75:20:2:3) as a developing solvent to afford N_b-demethyl-6-deoxy-16-acetoxy O^2-natafuranamin (**112**) (7 mg).

Fraction BMSF 200 (1.8 g), obtained on elution of the secondary column with 95% chloroform and 5% methanol, was again loaded onto a silica gel column. This column was eluted using hexane-acetone (0%-100%), and acetone-methanol (0%-100%) as a mobile phase. This mobile phase contained 1 ml diethylamine to provide fraction BMTF 83 (64.0 mg), which showed the presence of one major alkaloid along with several minor alkaloids. Preparative TLC of this fraction was performed using hexane-acetone-methanol-diethylamine (63:30:2:5) as a mobile phase in order to purify 6-deoxy-16-acetoxy O^{10}-natafuranamin (**113**) (3.5 mg).

Fraction BMSF 90-98 (1.0 g), obtained on elution of the secondary column with

80% hexane and 20% chloroform, was loaded into a silica gel column. This column was eluted using hexane-acetone (0%-100%) as a mobile phase to provide fraction BMTF 102 (449.3 mg). This fraction showed the presence of **114** as a major compound along with several minor compounds. Preparative TLC of this fraction was carried out using hexane-acetone-diethylamine (86:10:4) as a developing solvent to afford cycloprotobuxine-D (**114**) (4 mg).

Fraction BMSF 73 (2.9 g) obtained on elution of the secondary column with 84% hexane and 16% chloroform, was loaded into a silica gel column. This column was eluted with hexane-dichloromethane (0%-100%) and dichloromethane-methanol (0%-100%). The afforded fraction BMTF 50-59 (51.0 mg), which on analytical TLC using hexane-acetone-diethylamine (96:2:2) revealed the presence of **115** as a major compound along with several minor compounds. Prep. TLC of this fraction, using same mobile phase as that of analytical TLC yielded cycloprotobuxine-F **115** (4.8 mg).

Nb-Demethyl-6-deoxy-16-acetoxy O^2-natafuranamin (**112**): a colorless amorphous solid 7.4 mg; UV ($CHCl_3$) λ_{max} 234 nm; IR ($CHCl_3$): 1643, 1711, and 1089 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR APT ($CDCl_3$, 100 MHz), δ: see Table 2.1; HREIMS [M^+ m/z 562.3402 (observed), $C_{34}H_{46}N_2O_5$, 562.3407 (calcd)].

6-Deoxy-16-acetoxy O^{10}-natafuranamin (**113**): white amorphous powder 4.22 mg; UV (MeOH) λ_{max} 224 nm; IR ($CHCl_3$): 1645, 1709, and 1095 cm^{-1}; ^{1}H-NMR (acetone-d_6, 400 MHz) and ^{13}C-NMR APT (acetone-d_6,100 MHz), δ: see Table 2.2; HREIMS [M^+ m/z 560.3610 (observed), $C_{35}H_{48}N_2O_4$, 560.3614 (calcd)].

Cycloprotobuxine-D (**114**): white amorphous powder 3 mg; UV ($CHCl_3$) λ_{max} 202 nm; IR ($CHCl_3$): 3399 cm^{-1}; ^{1}H-NMR ($CDCl_3$, 400 MHz) and ^{13}C-NMR APT ($CDCl_3$, 100 MHz), δ: see Table 2.3; EIMS [m/z 386 (observed), $C_{35}H_{48}N_2O_4$, 386.37 (calcd)].

Cycloprotobuxine-F (**115**): yellow colored gum 4.8; UV (MeOH) λ_{max} 202 nm; IR ($CHCl_3$) 3411 cm^{-1}; ^{1}H-NMR (pyridine-d_6, 400 MHz) and ^{13}C-NMR APT (pyridine-d_6,100 MHz), δ: see Table 2.4; EIMS [m/z 386 (observed), $C_{35}H_{48}N_2O_4$, 386.37 (calcd)].

2.3.4 ACETYLCHOLINESTERASE INHIBITION ASSAY

The isolated compounds (**112-115**) were evaluated for acetylcholinesterase inhibition activity by using the Ellman method with slight modifications.[16] This method is based on the measurement of the enzyme activity by monitoring thiocholine formation which conjugate with DTNB (5, 5 dithiobis-2-nitrobenzoate) to form thiocholine-DTNB adduct. This adduct has a maximum absorption at 406 nm. The percentage inhibition was calculated by $[(A_O – A_I) / A_O] \times 100$ where A_O is the absorbance of the blank, and A_I the average absorbance values for each various concentrations of a tested compound. The IC_{50} concentration required for these compounds to inhibit 50% of the enzyme activity was determined from the chart plot between the compound concentrations and percentage inhibition. A mixture of enzyme (2.5 mU/mL), 100 mM phosphate buffer at pH 8.0 (126 µL), and 0.01 M DTNB (50 µL), was incubated at room temperature with the tested compounds for 30 minutes. Formation of the thiocholine-DTNB adduct was inducted by the addition of 20 µL of 0.075 M acetylthiocholine. The formation of thiocholine-DTNB adduct was measured at 406 nm 3 minutes after the addition of acetylthiocholine.

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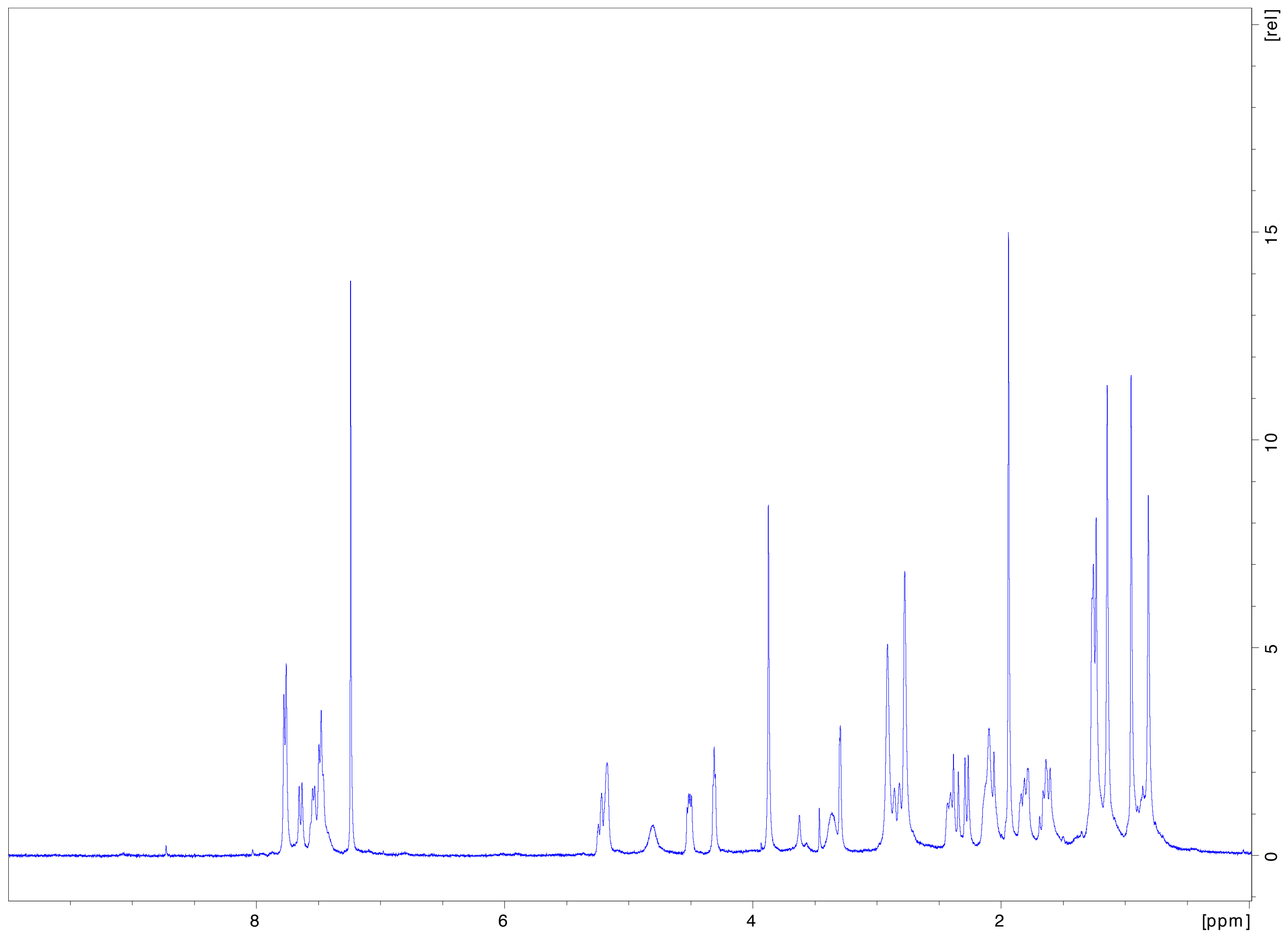
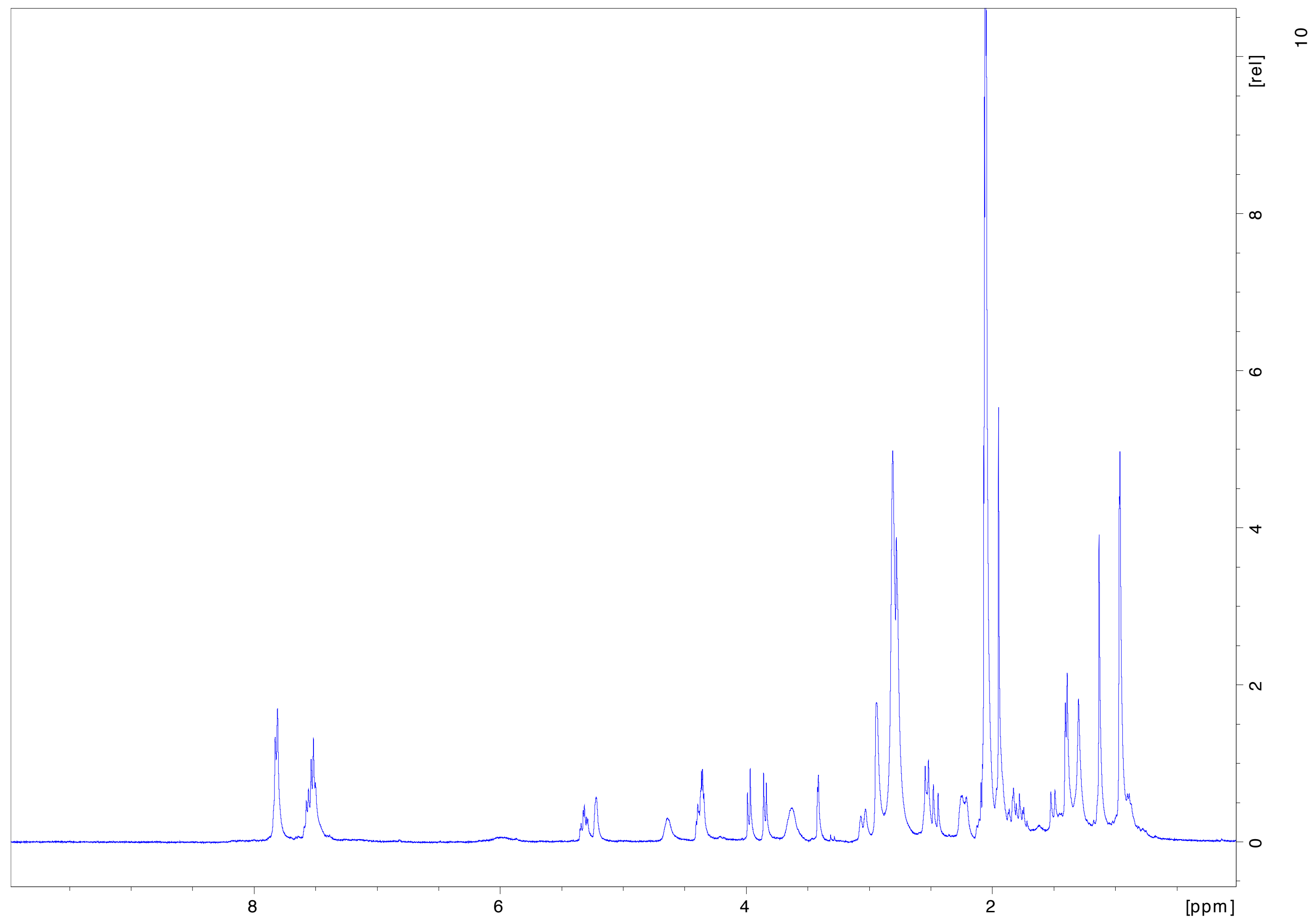
CONCLUSION

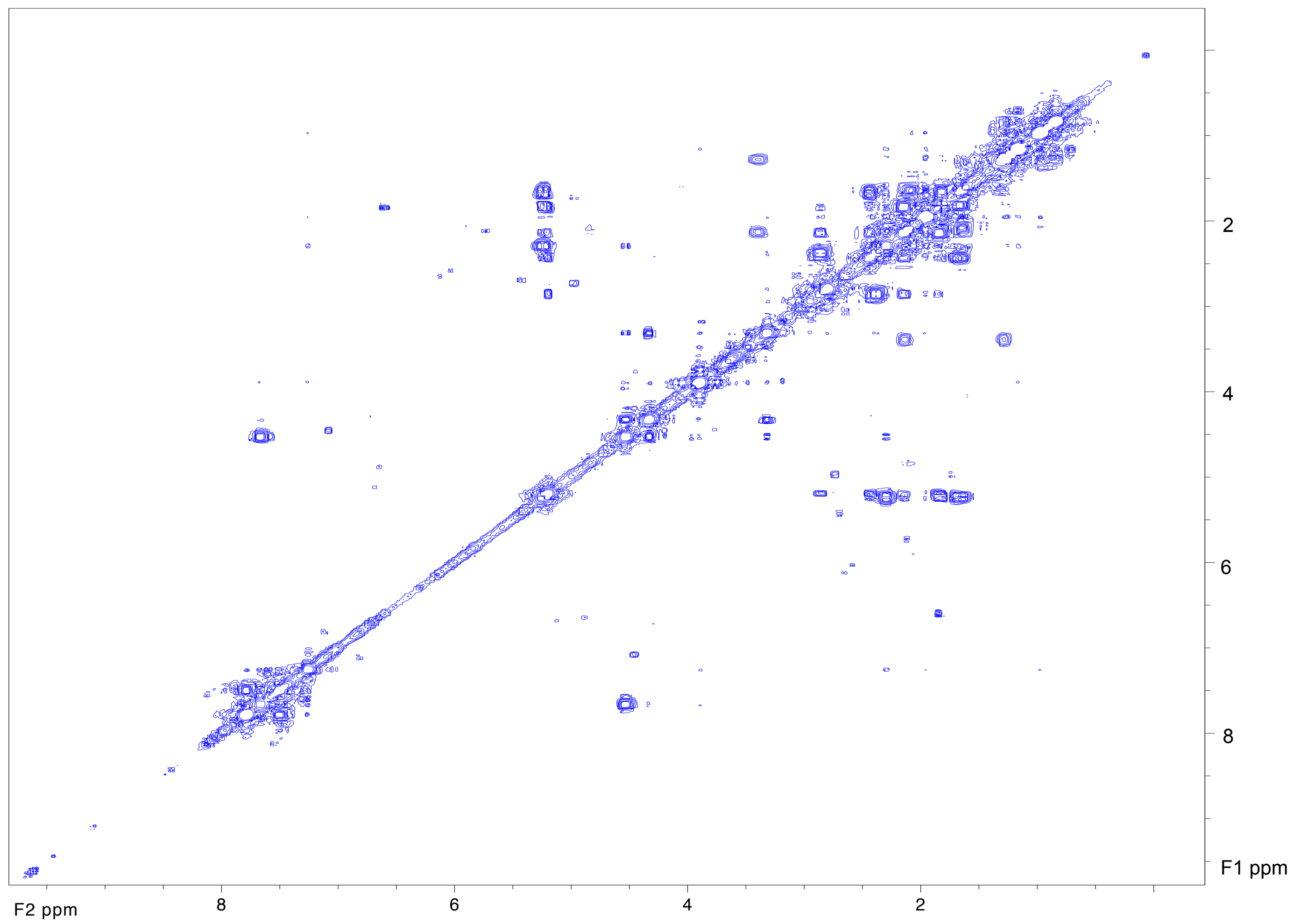
In summary, phytochemical studies on the bark extract of *B. macowanni* resulted in the isolation of two novel steroidal alkaloids, N_b-demethyl-6-deoxy-16-acetoxy O^2-natafuranamin (**112**) and 6-deoxy-16-acetoxy O^{10}-natafuranamin (**113**), along with two known steroidal alkaloids cycloprotobuxine-D **(114)** and cycloprotobuxine-F (**115**), which were isolated for the first time from this plant. Compounds **112** and **113** had a tetrahydrofuran ring incorporated in their structure. A few *Buxus* alkaloids belonging to this class have been reported in literature. Tetrahydrofuran rings may be biosynthesized in nature from $\Delta^{1\text{-}2}$ 9(10→19) *abeo* non-conjugated alkaloids containing a hydroxyl group at C-31 as outlined on chapter 1 page 33 (introduction). Moreover, compound **112** exhibited significant activity in the AChE inhibitory assay (IC_{50} = 4.7 μM). Compound **113** also displayed enhanced AChE inhibitory activity compared to compounds **114** and **115**, which showed low AChE inhibitory activity. The AChE inhibitory activity of the isolated compounds **112-115** determined via this assay, are in agreement with results previously reported for O^2-natafuranimne, and the other *Buxus* alkaloids. As a result of this, further structure-activity relationship studies on compounds **112** and **113** are demanded to improve its bioactivity.



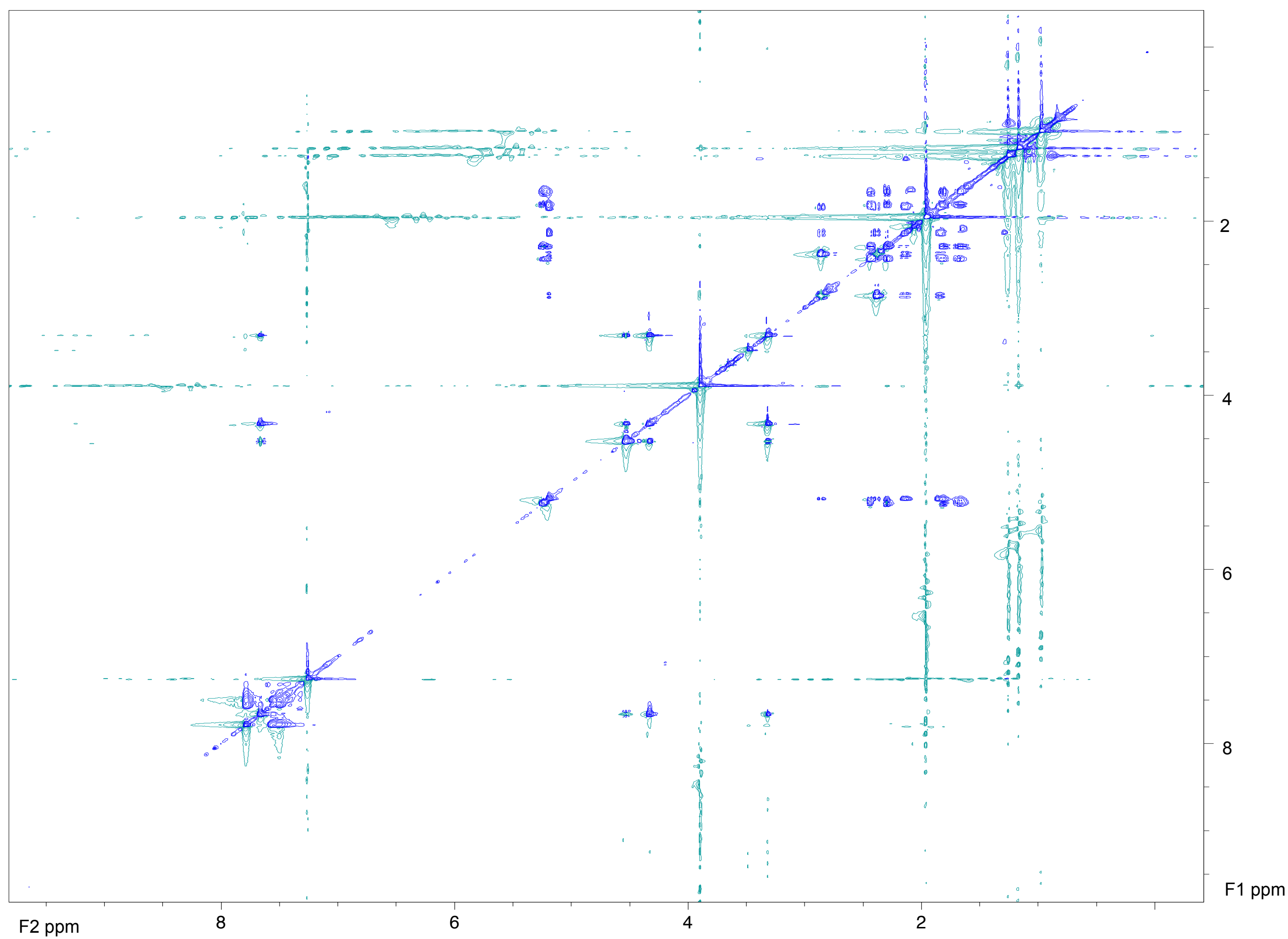
A1. ^{1}H-NMR spectrum of compound (**112**) in $CDCl_3$.



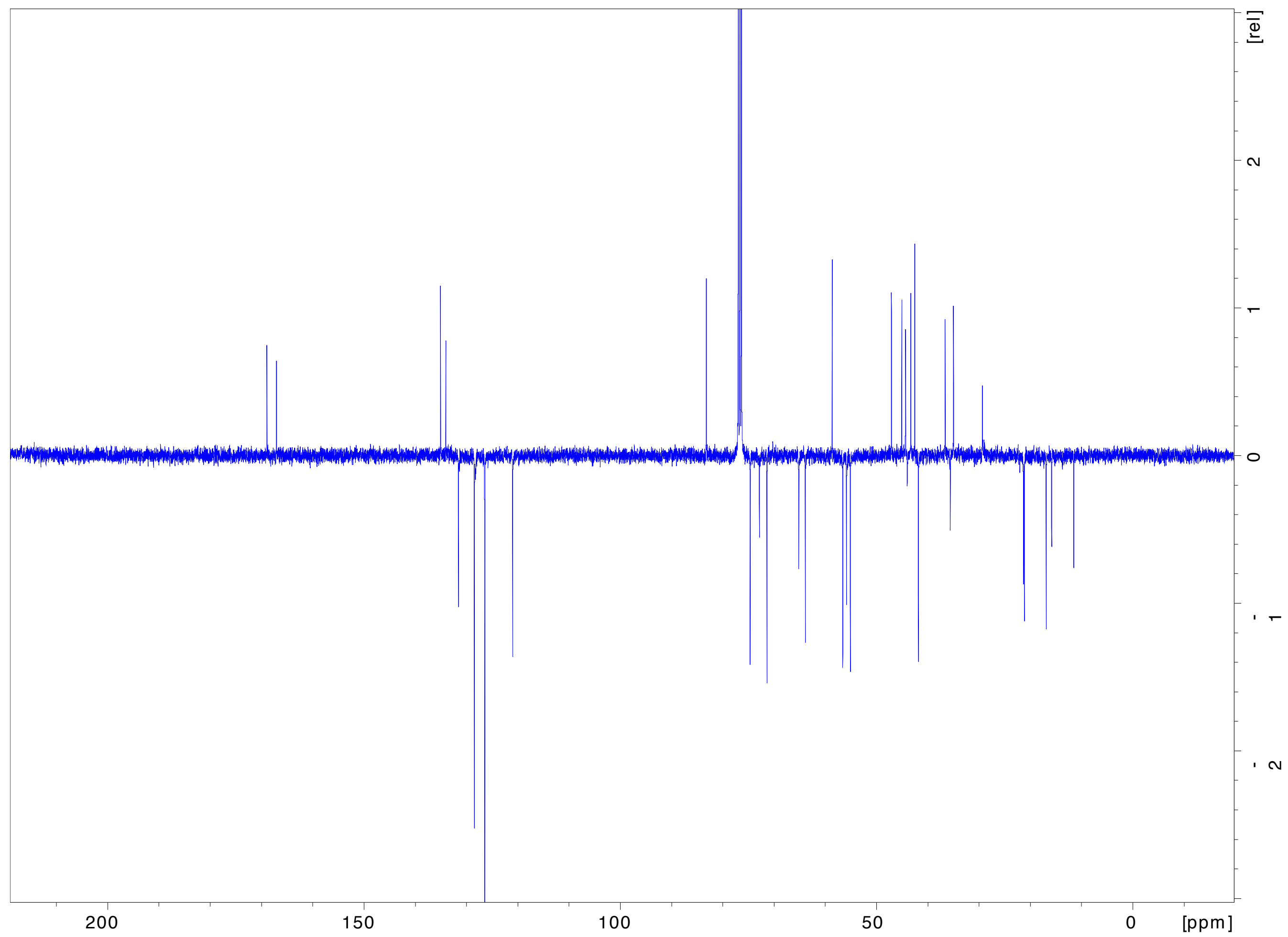
A2. ^{1}H-NMR spectrum of compound (**112**) in acetone d_6.



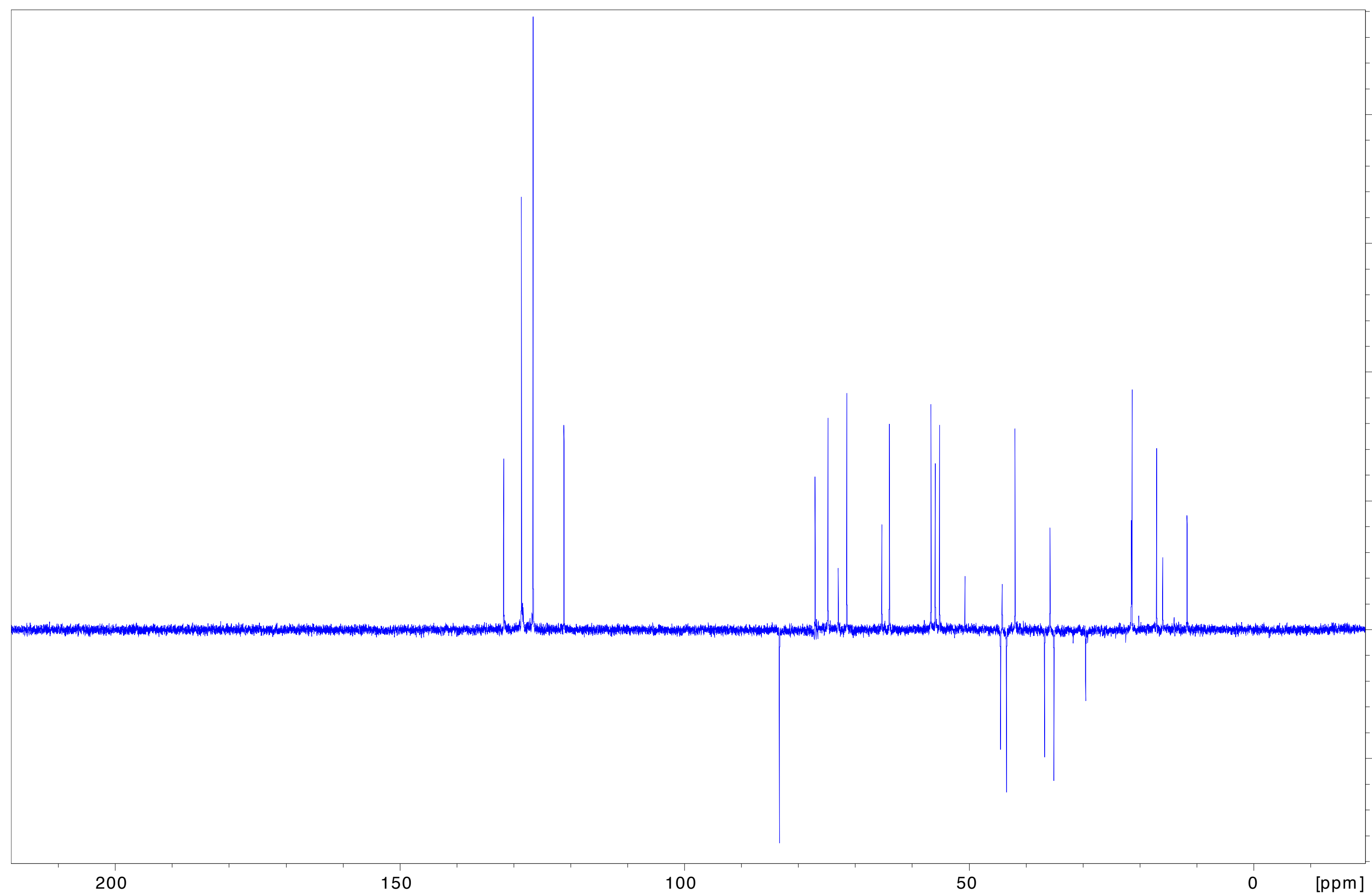
A3. COSY-45° spectrum of compound (**112**) in $CDCl_3$.



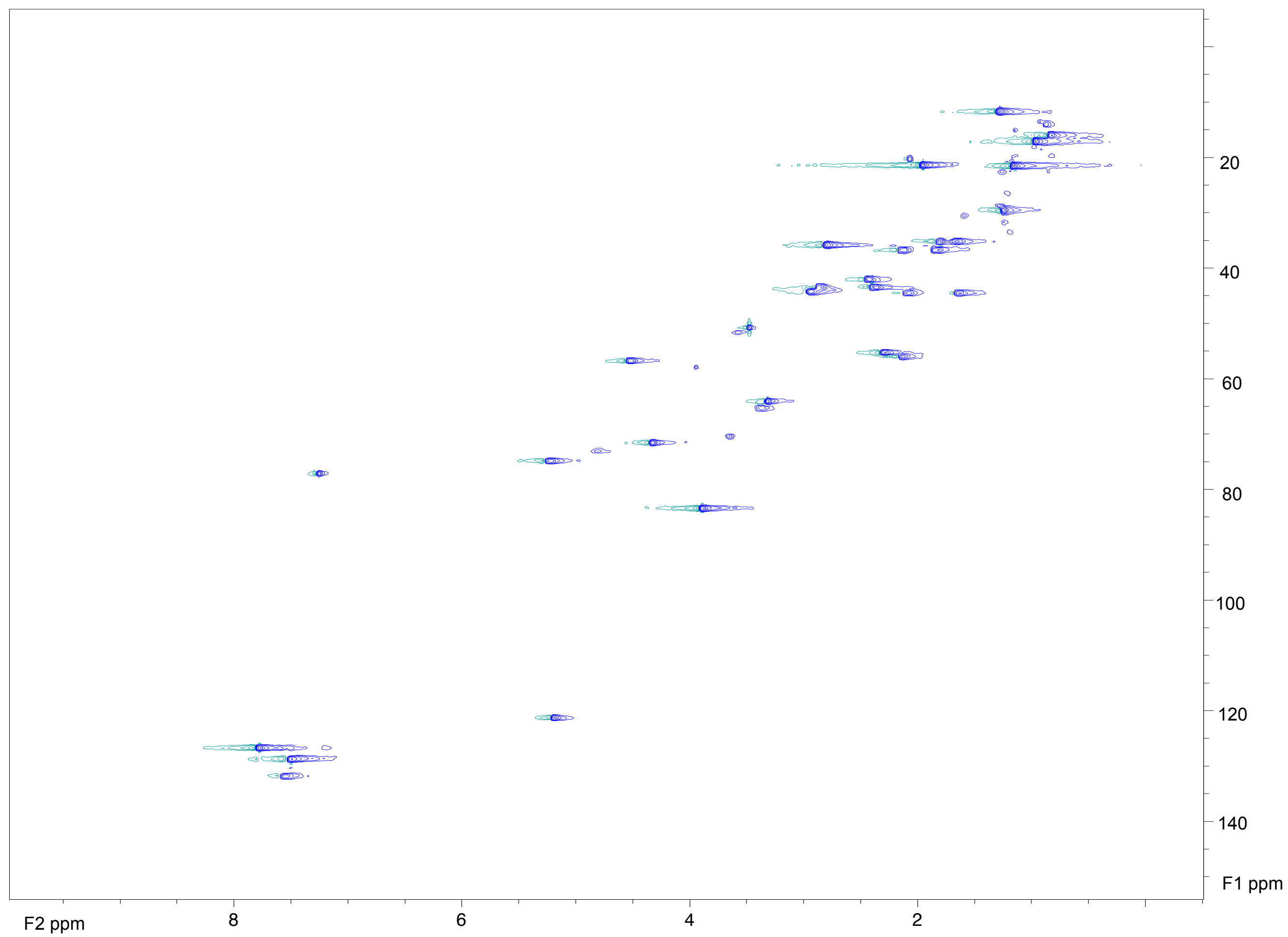
A4. TOCSY spectrum of compound (**112**) in $CDCl_3$.



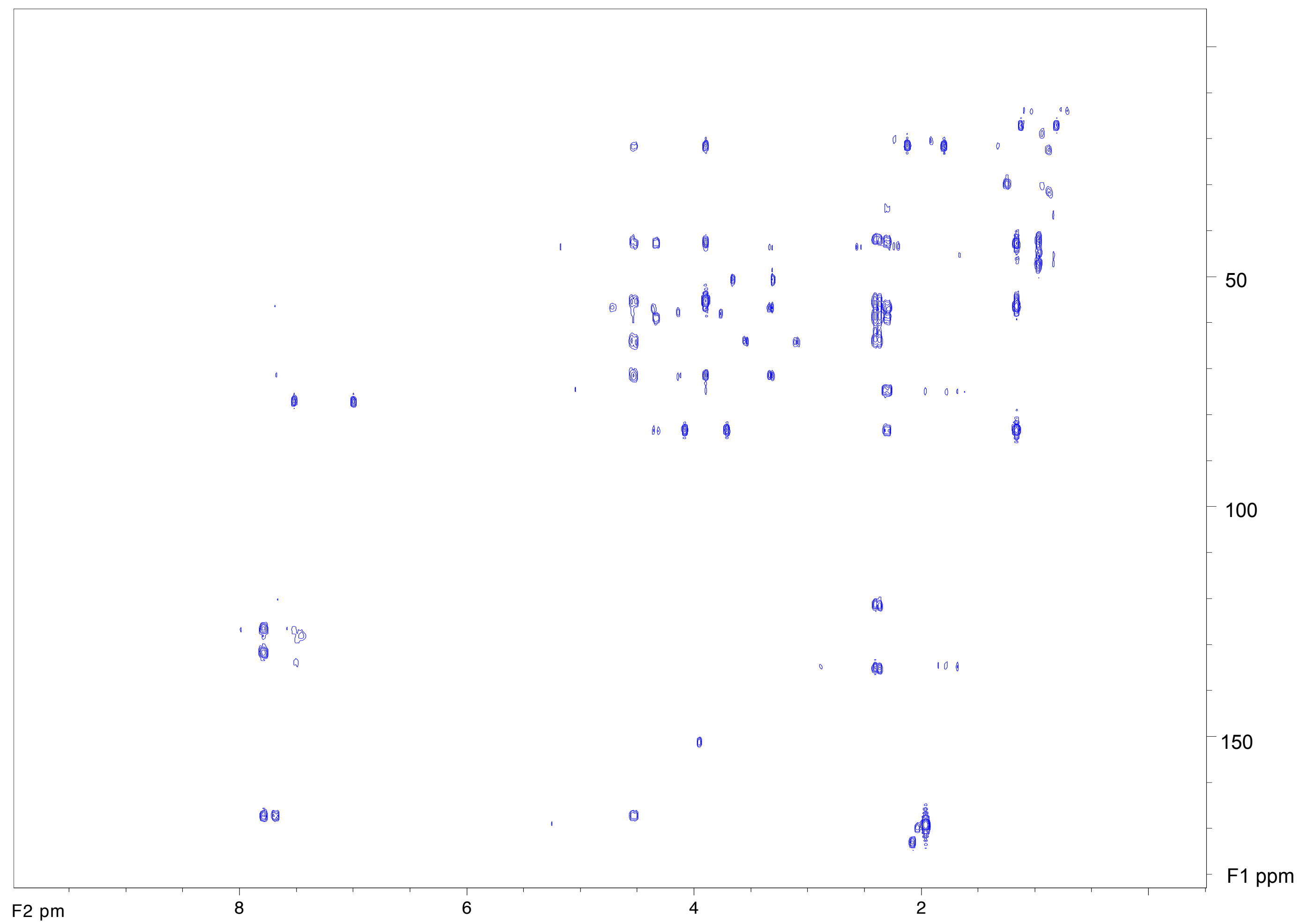
A5. APT spectrum of compound (**112**) in $CDCl_3$.



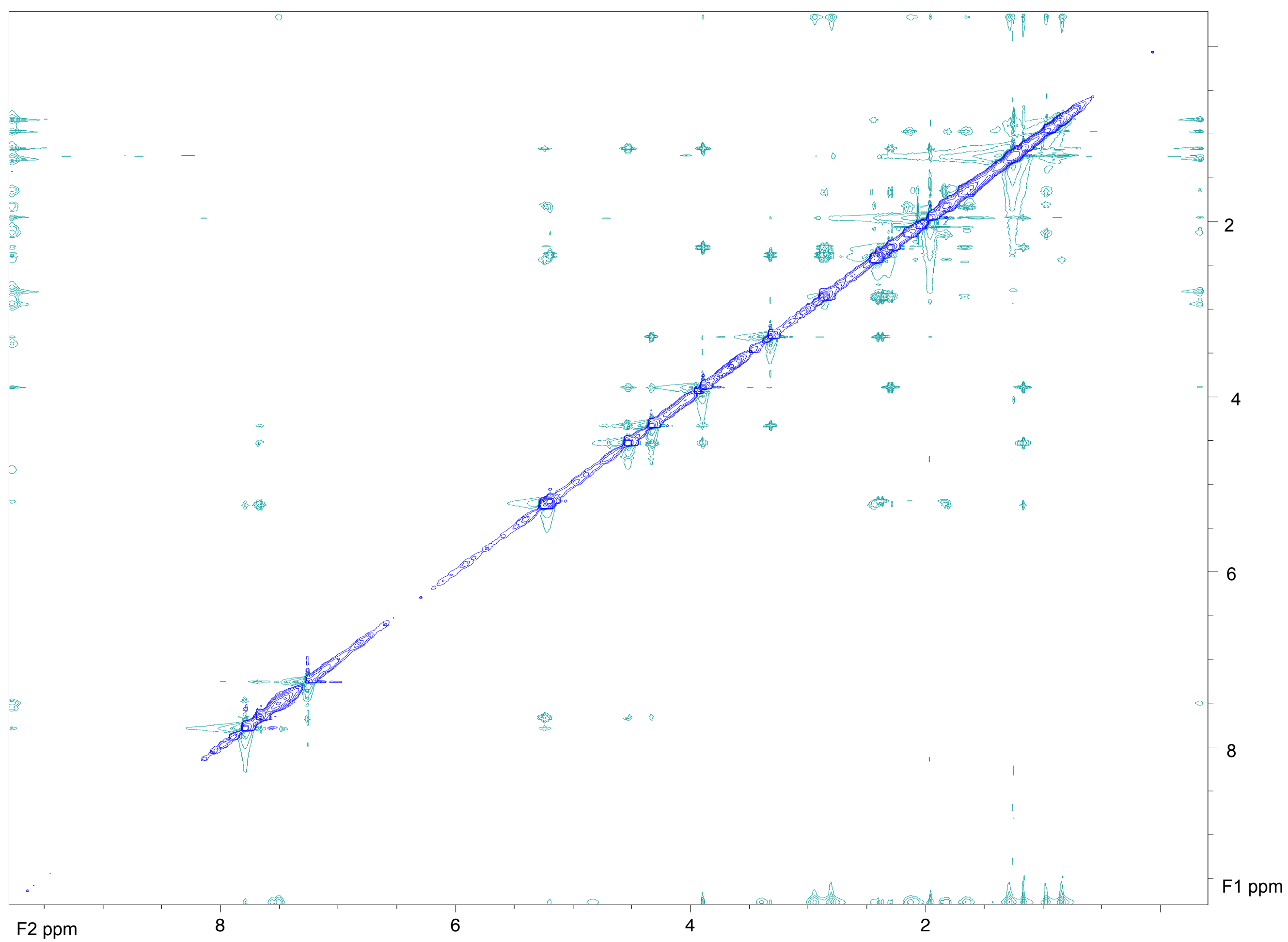
A6. DEPT-135 spectrum of compound (**112**) in $CDCl_3$.



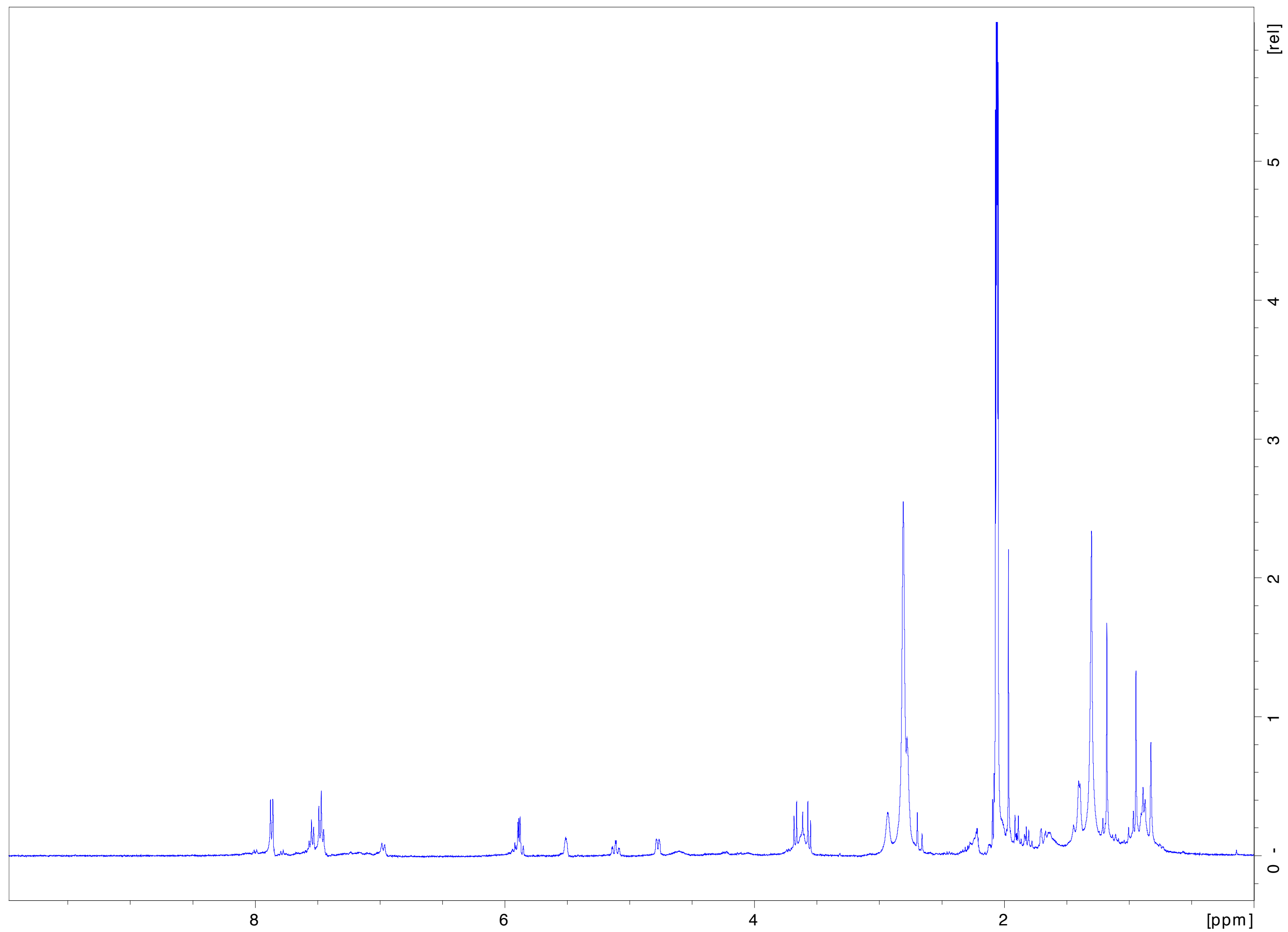
A7. HSQC spectrum of compound (**112**) in $CDCl_3$.



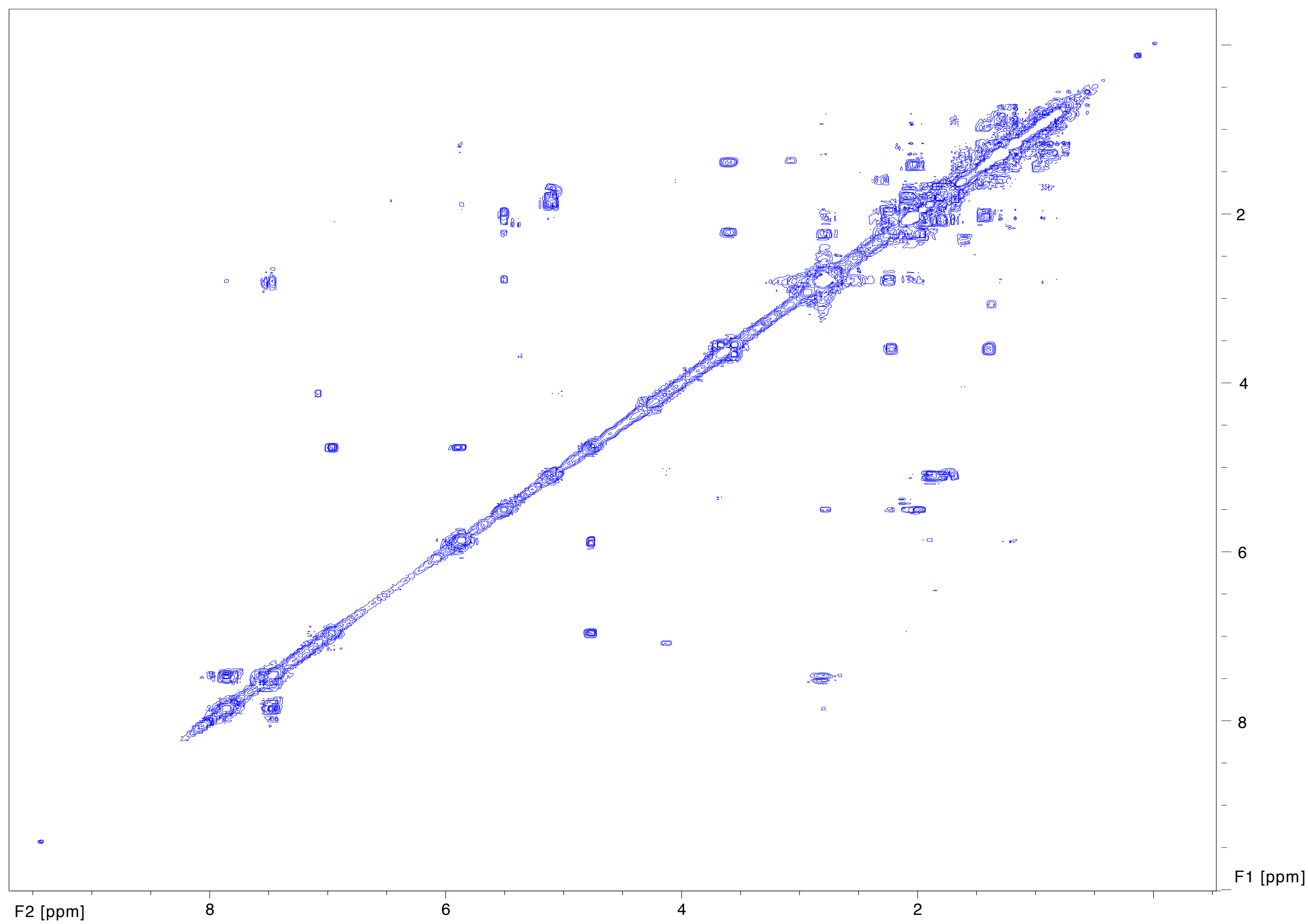
A8. HMBC spectrum of compound (**112**) in $CDCl_3$.



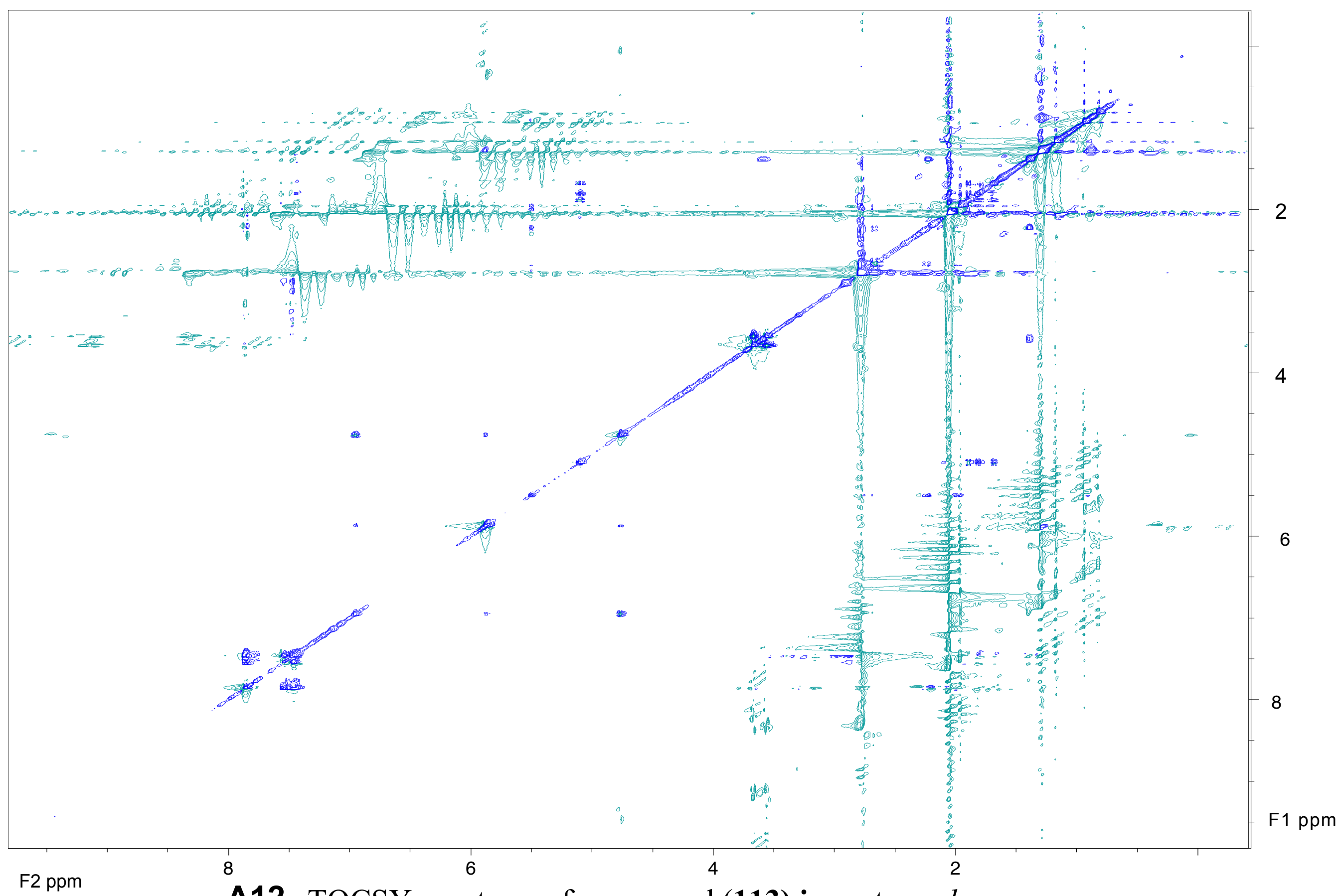
A9. NOESY spectrum of compound (**112**) in $CDCl_3$.



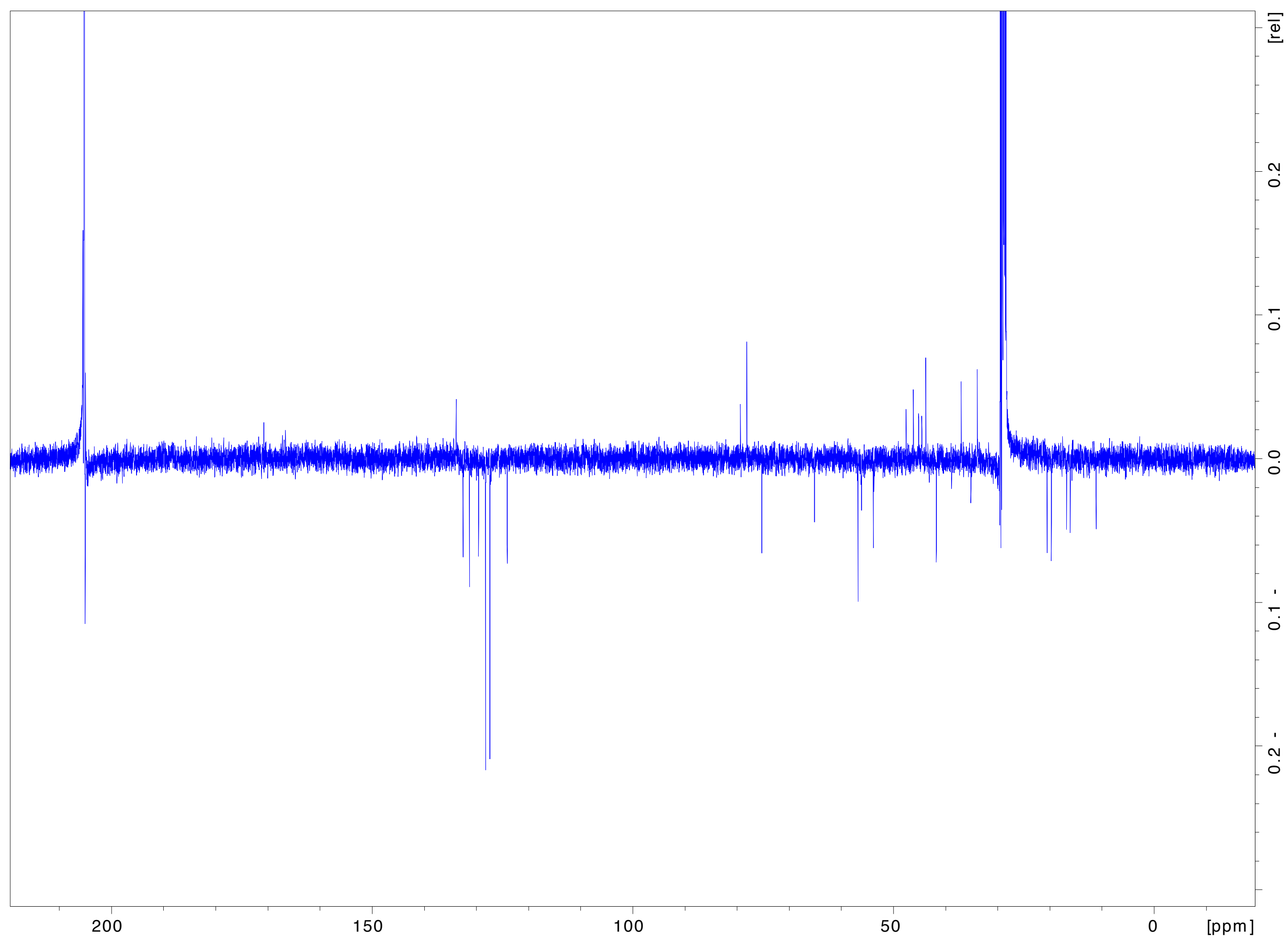
A10. ^{1}H-NMR spectrum of compound (**113**) in acetone-d_6 .



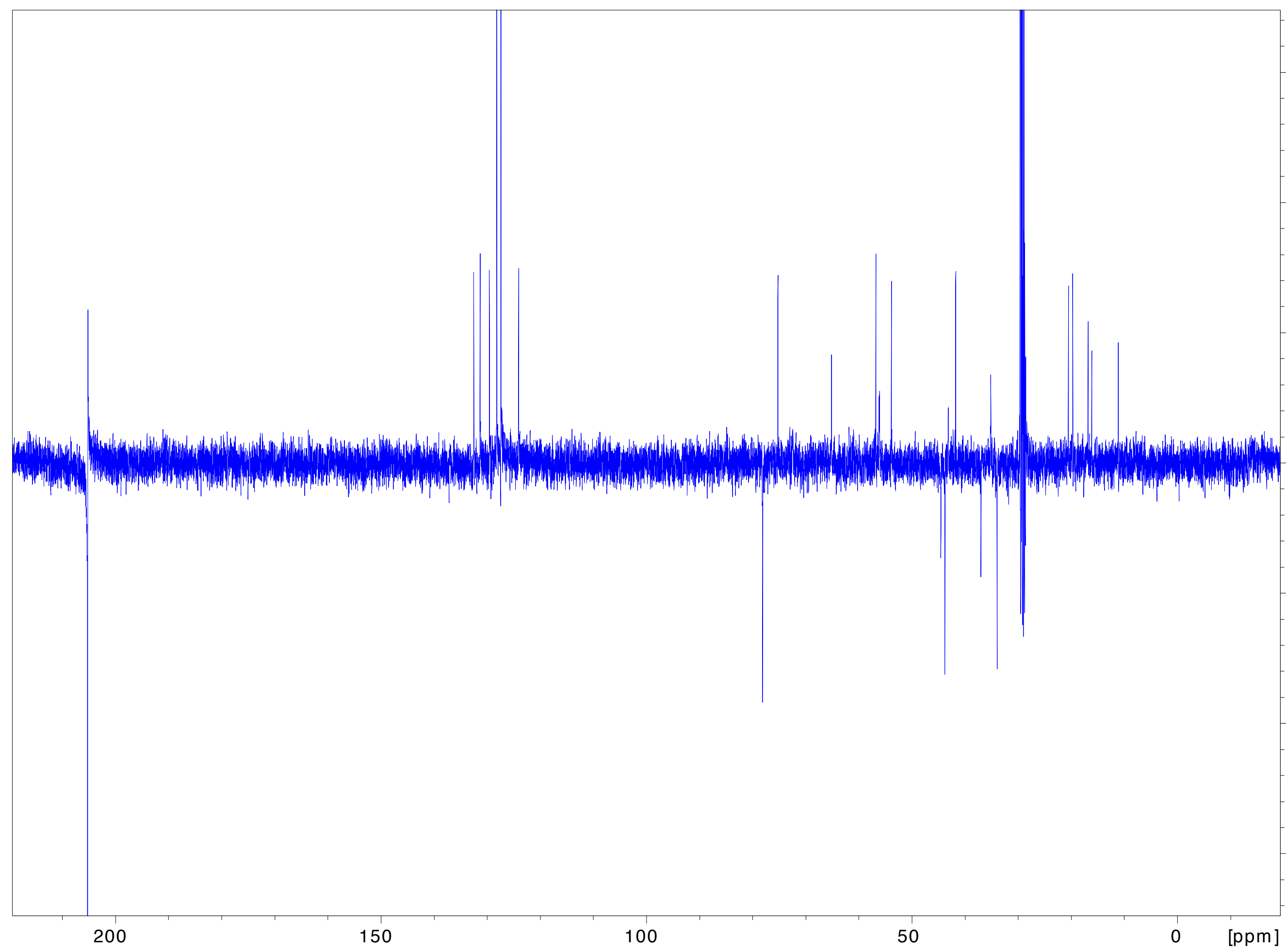
A11. COSY-45° spectrum of compound (**113**) in acetone-d_6.



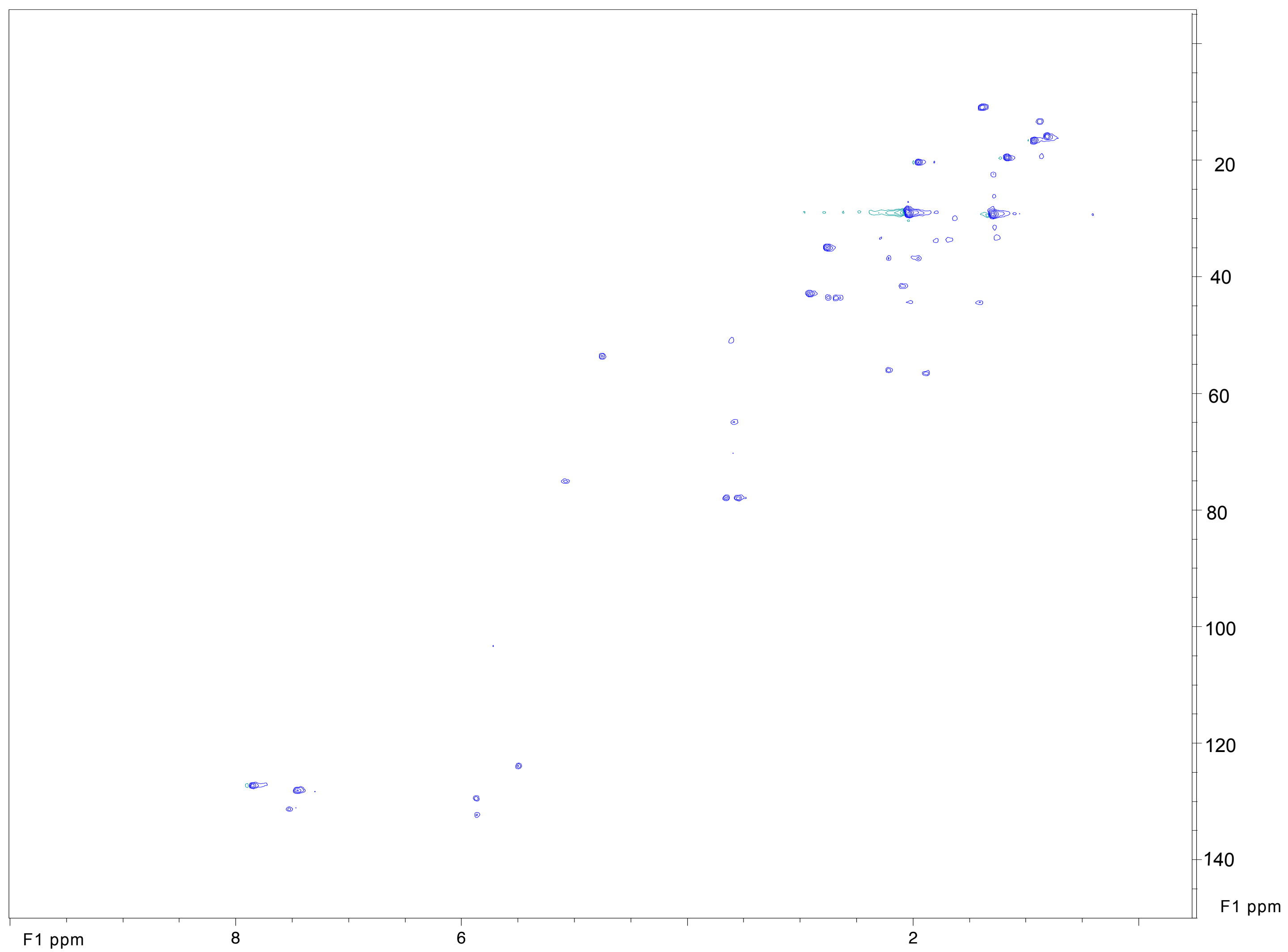
A12. .TOCSY spectrum of compound (**113**) **i**n acetone-d_6 .



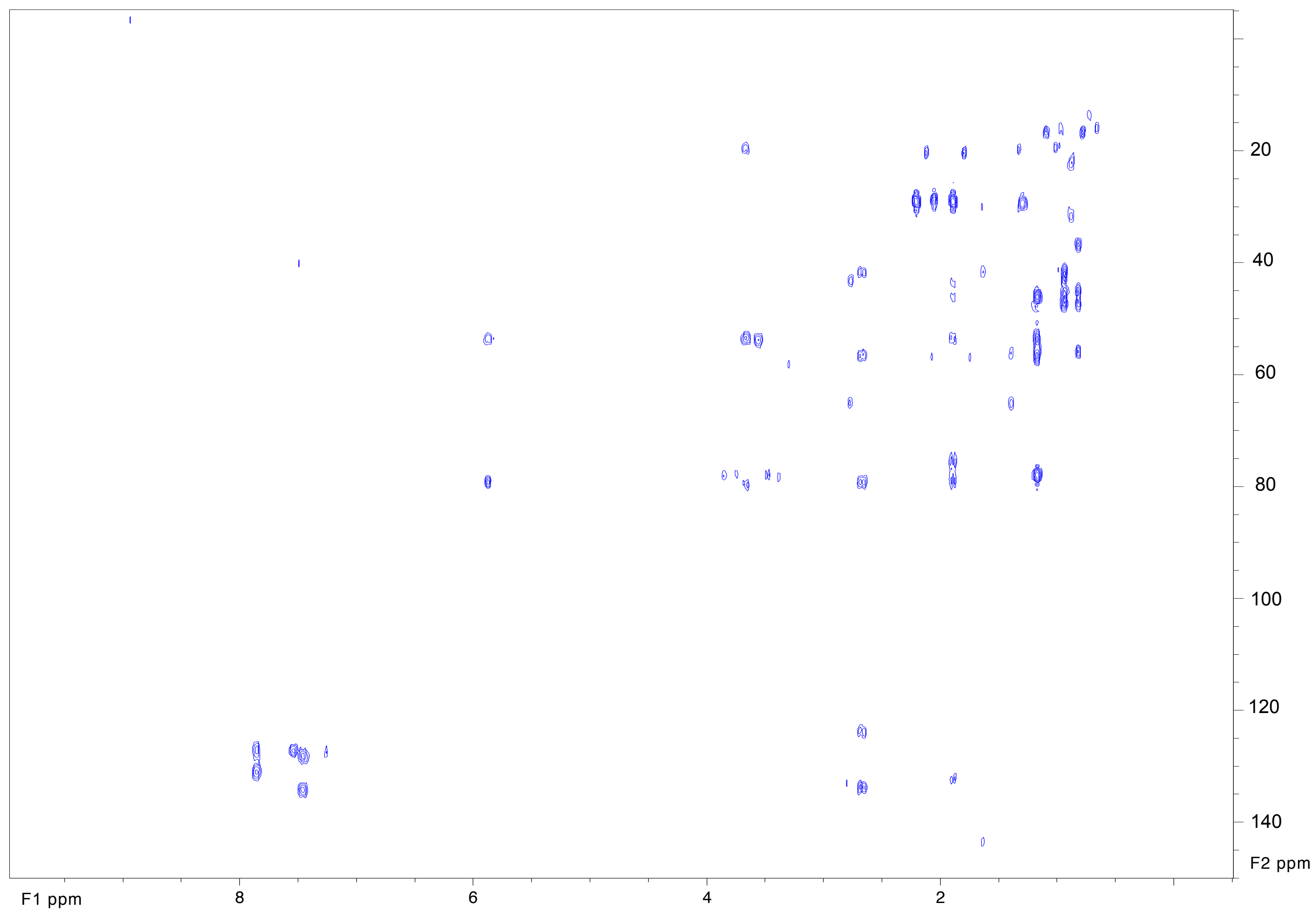
A13. APT spectrum of compound (**113**) in acetone-d_6 .



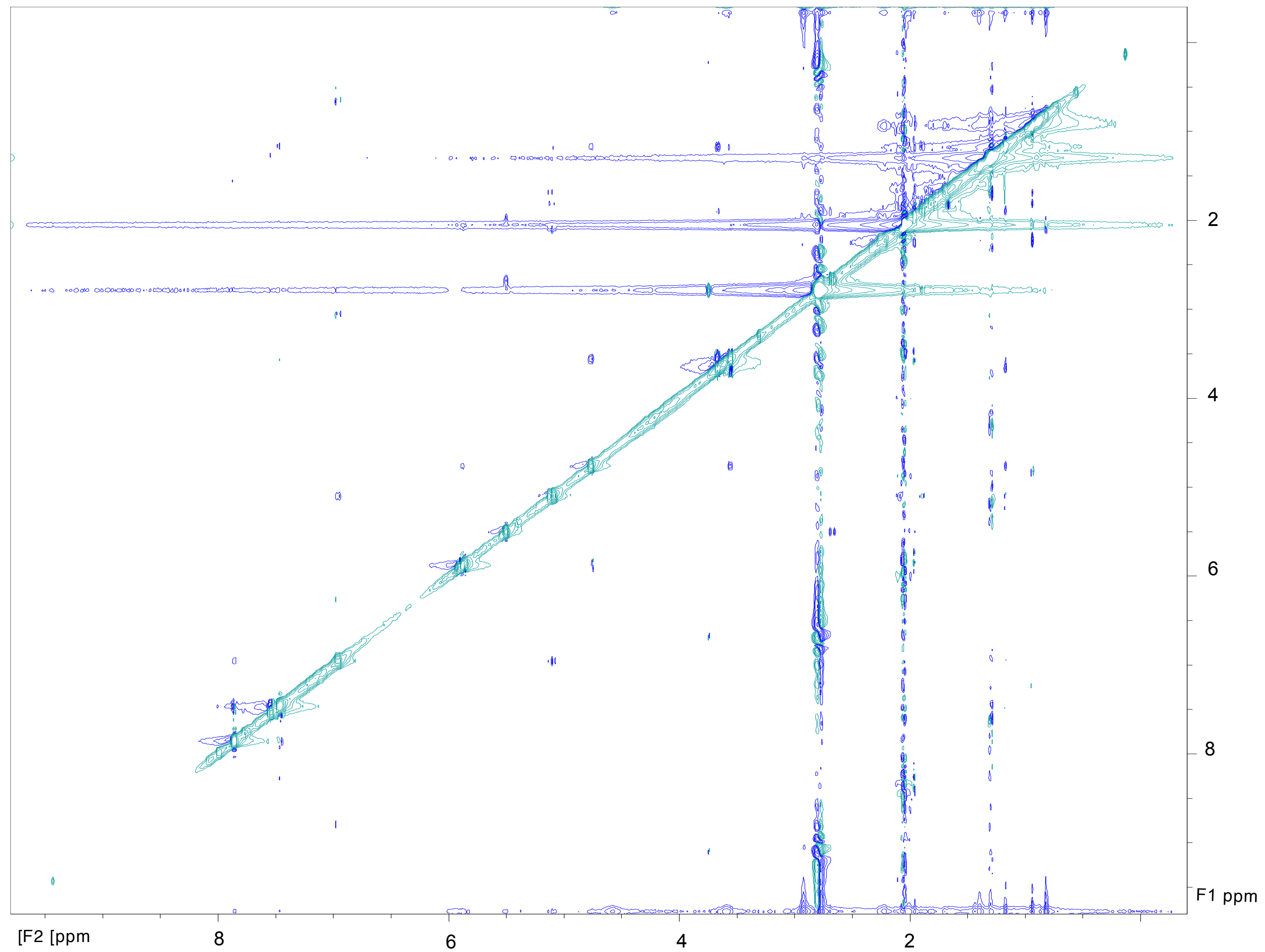
A14. DEPT-135 spectrum of compound (**113**) in acetone-d_6.



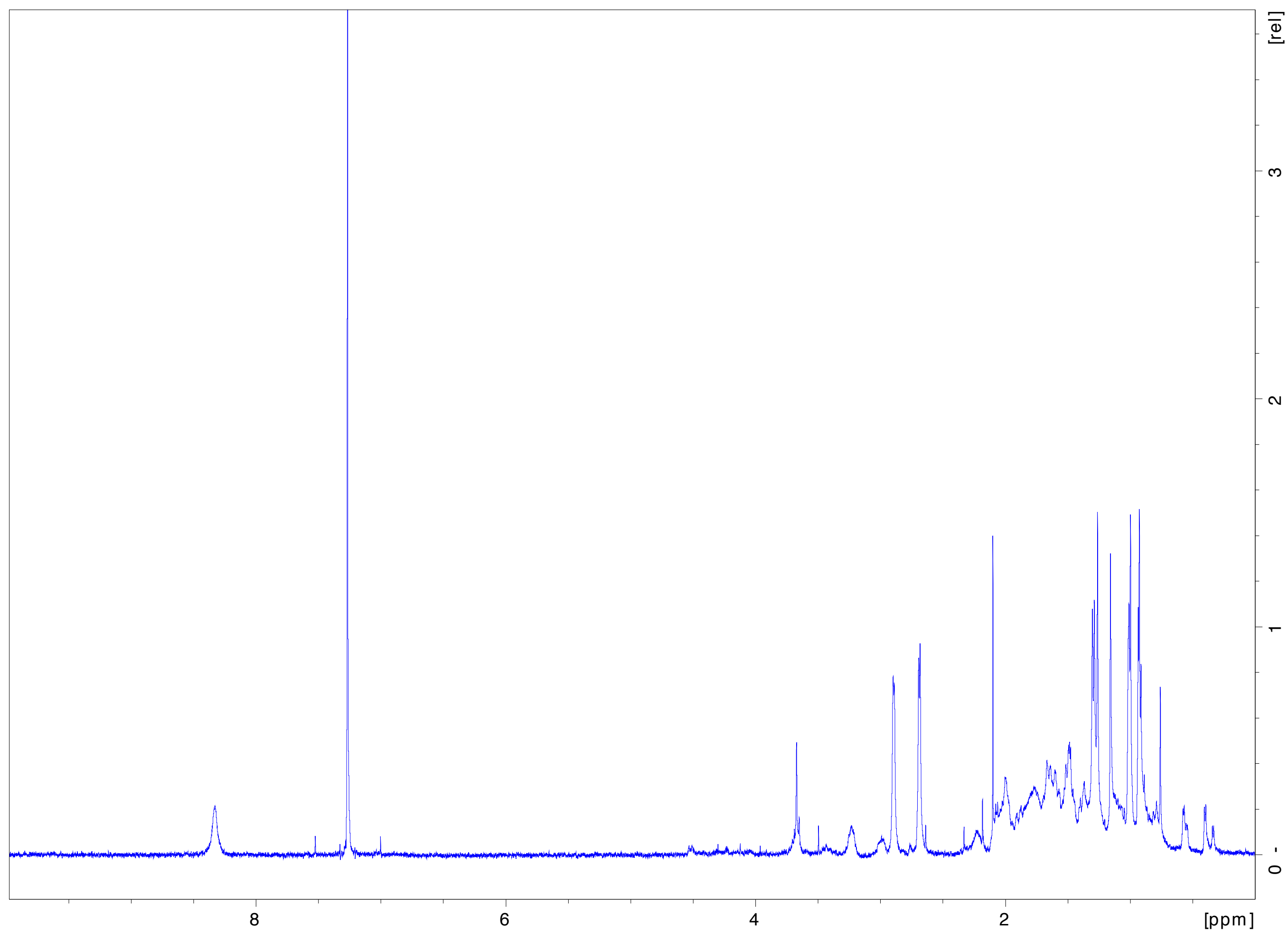
A15. HSQC spectrum of compound (**113**) in acetone-d_6.



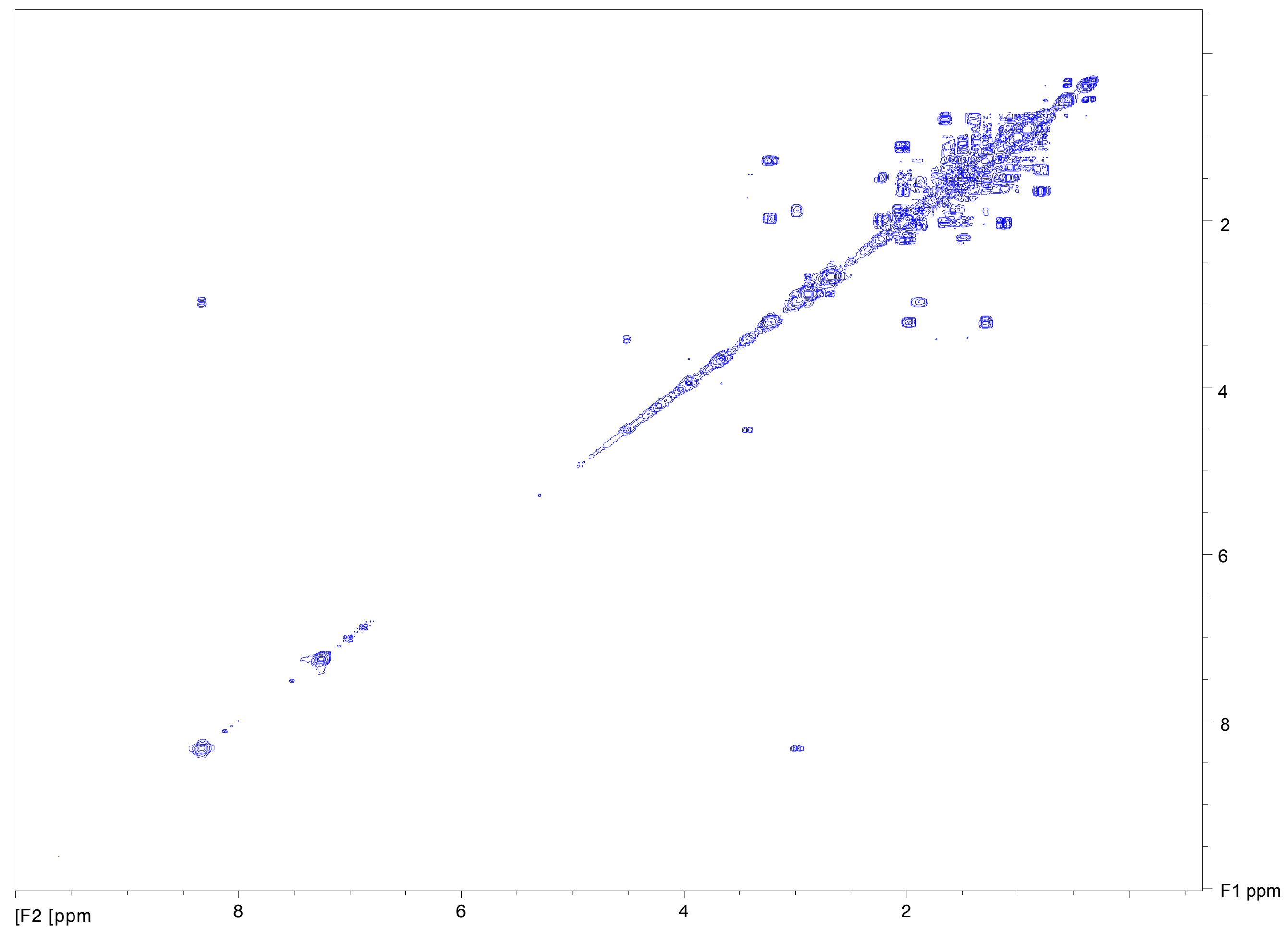
A16. HMBC spectrum of compound (**113**) in acetone-d_6.



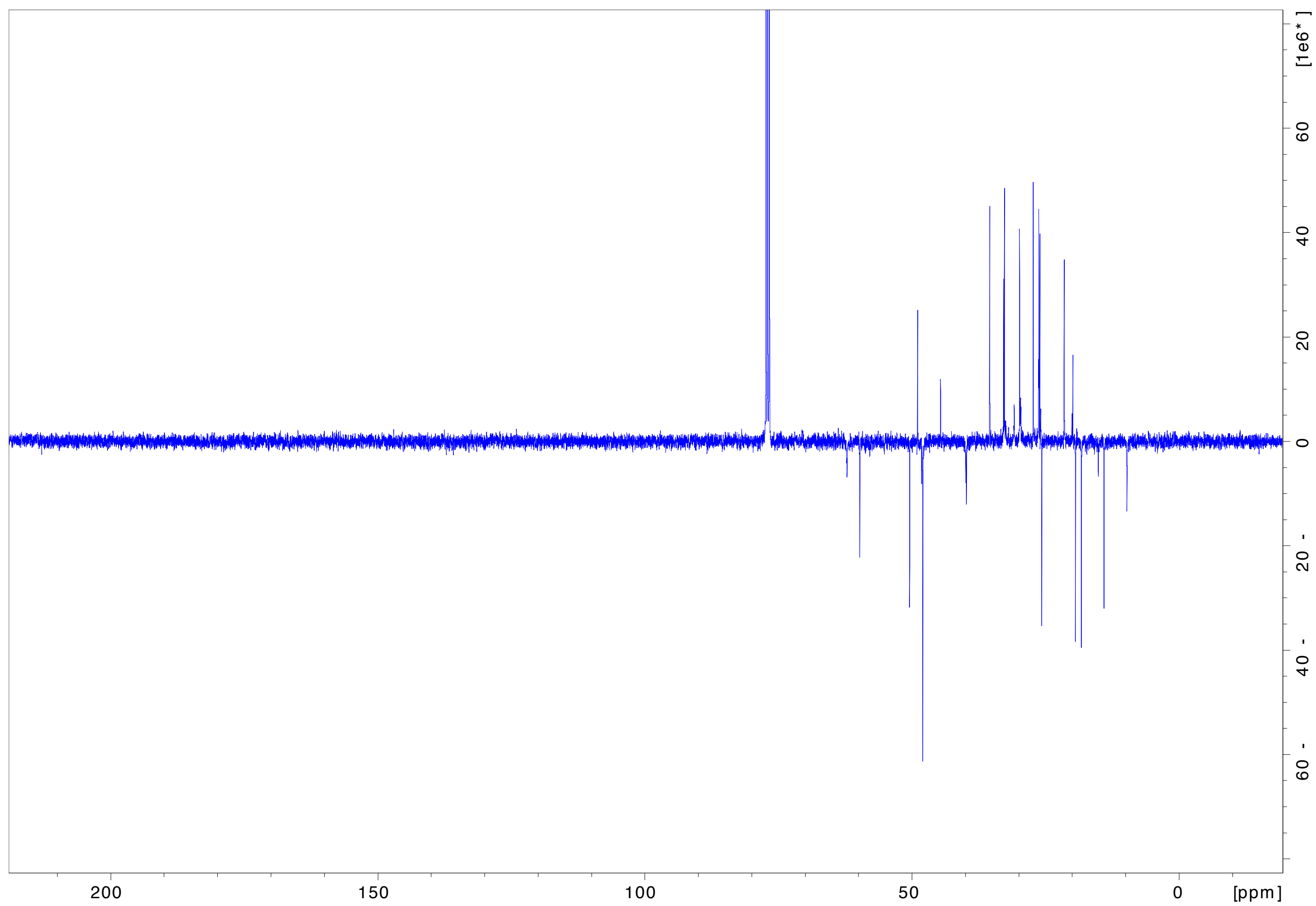
A17. NOESY spectrum of compound (**113**) in acetone-d_6.



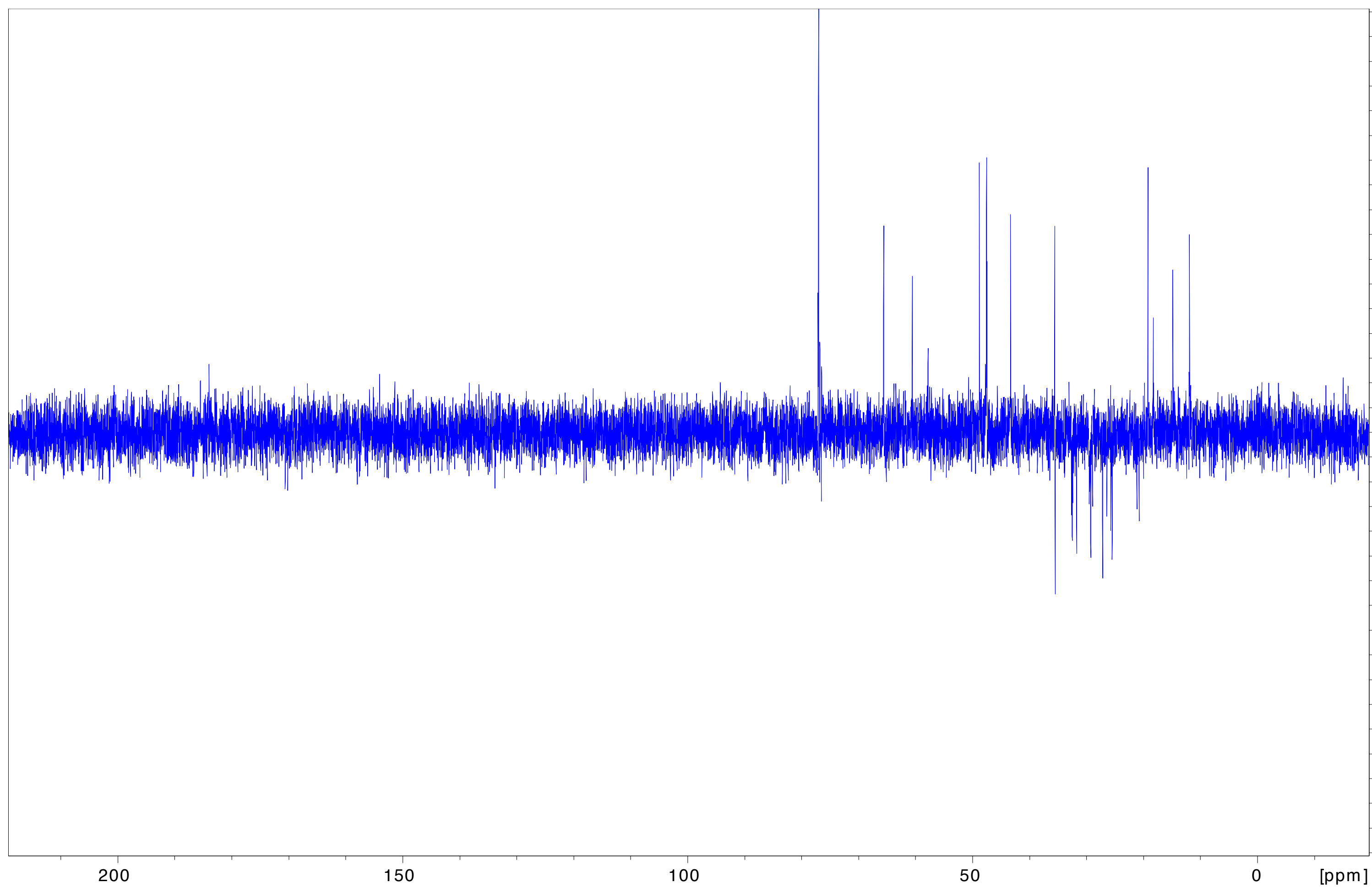
A18. ^{1}H-NMR spectrum of compound (**114**) in $CDCl_3$.



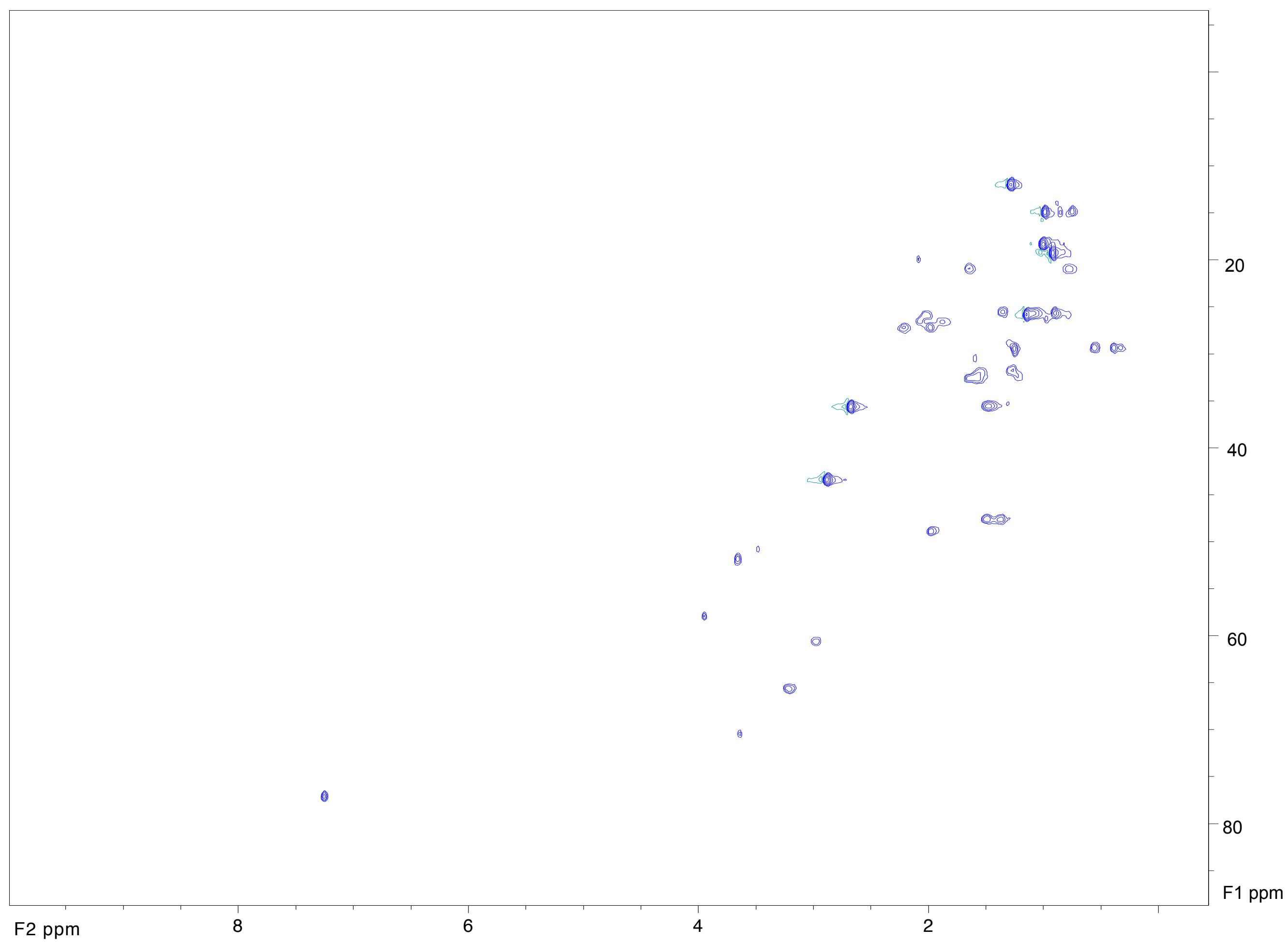
A19. COSY-45° spectrum of compound (**114**) in $CDCl_3$.



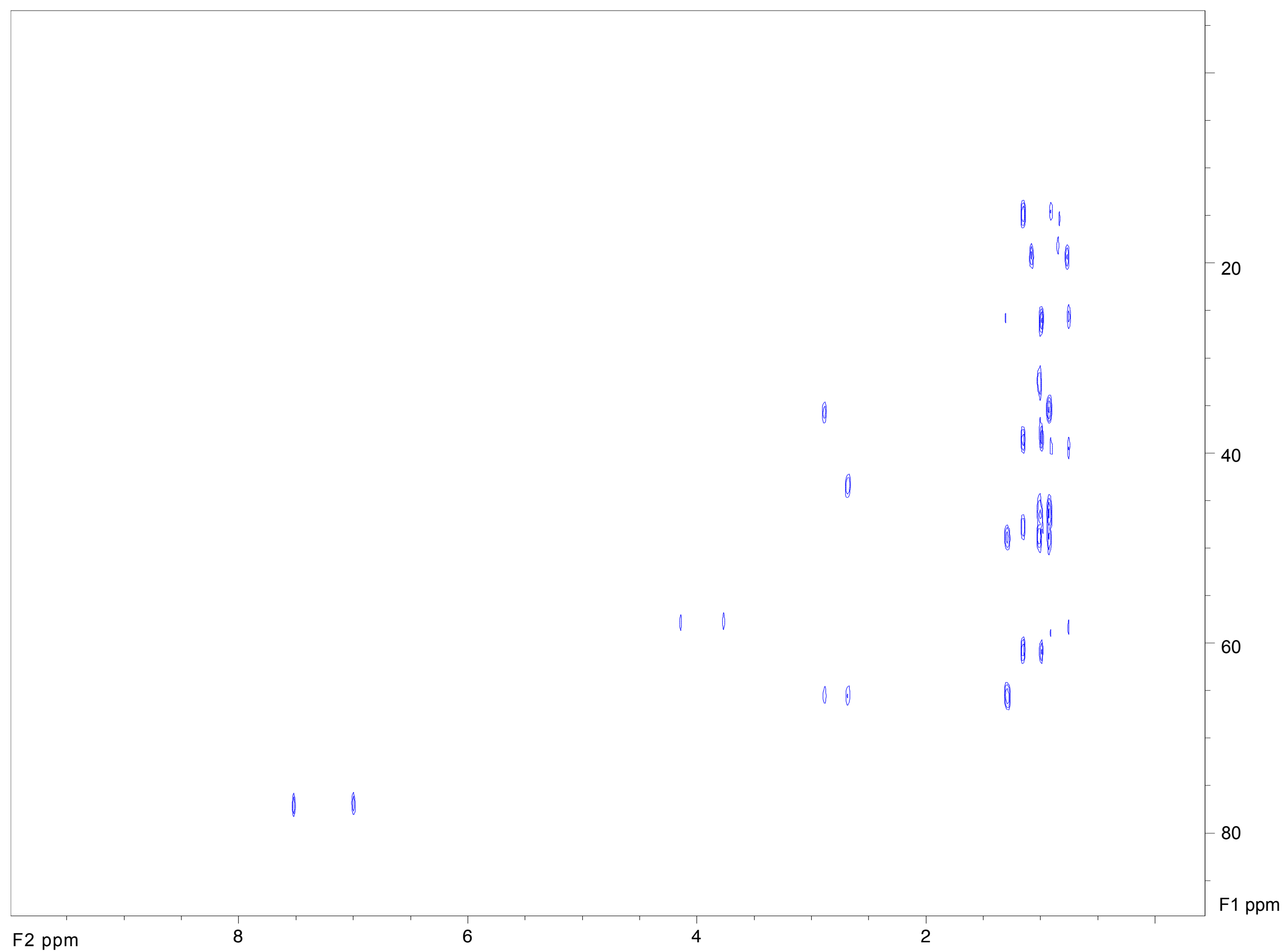
A20. APT spectrum of compound (**114**) in $CDCl_3$.



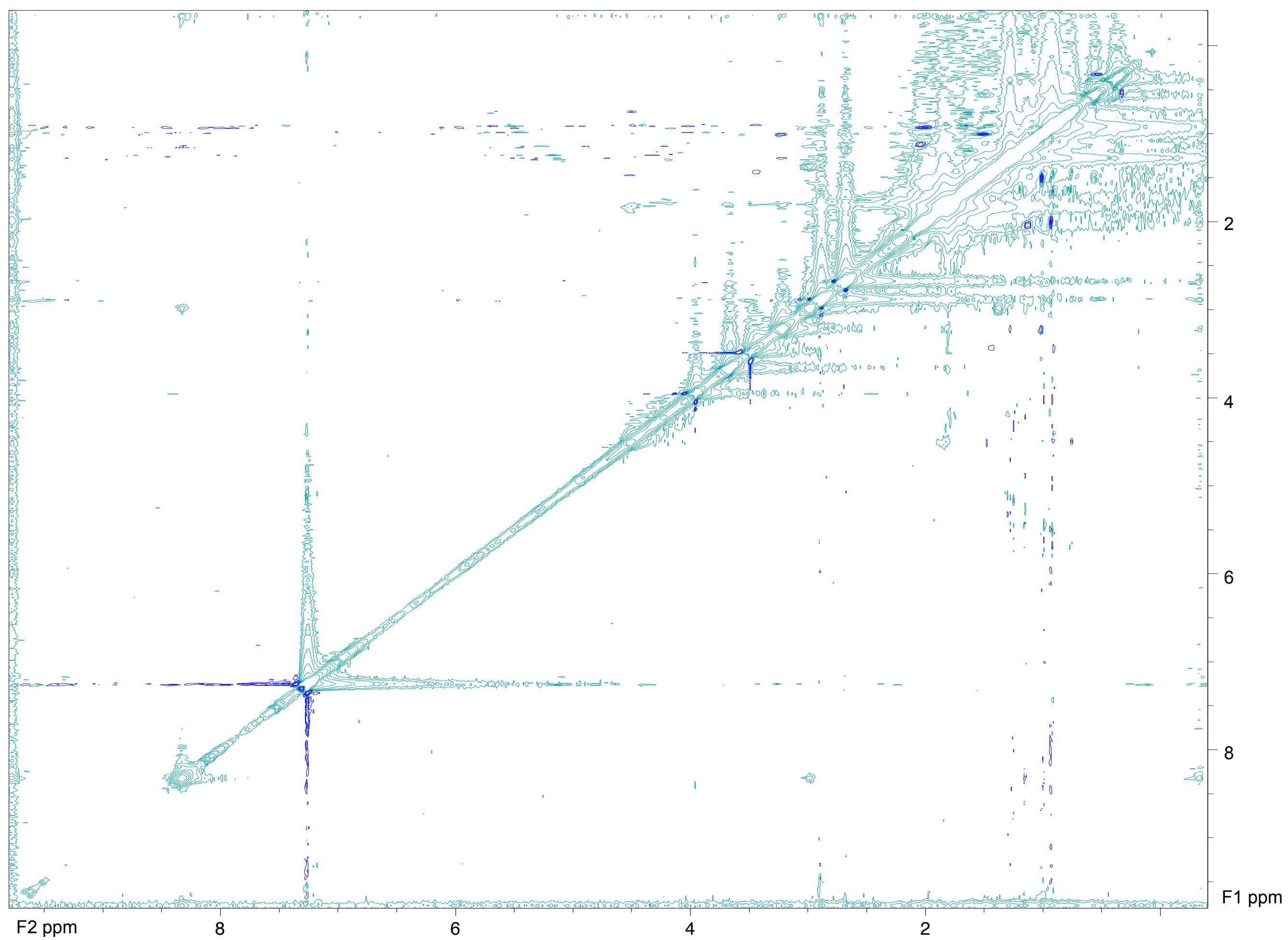
A21. DEPT-135 spectrum of compound (**114**) in $CDCl_3$.



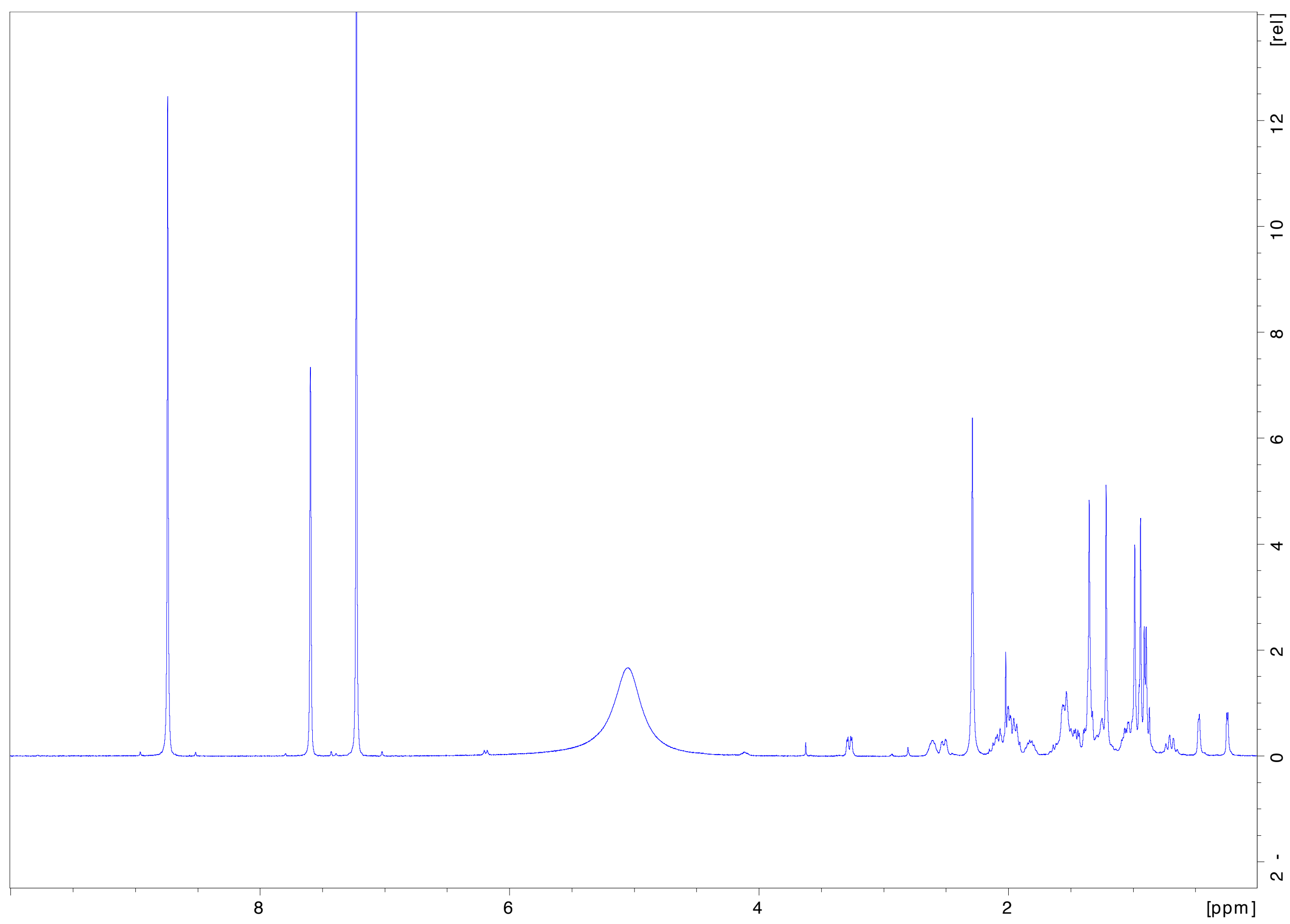
A22. HSQC spectrum of compound (**114**) in $CDCl_3$ ·



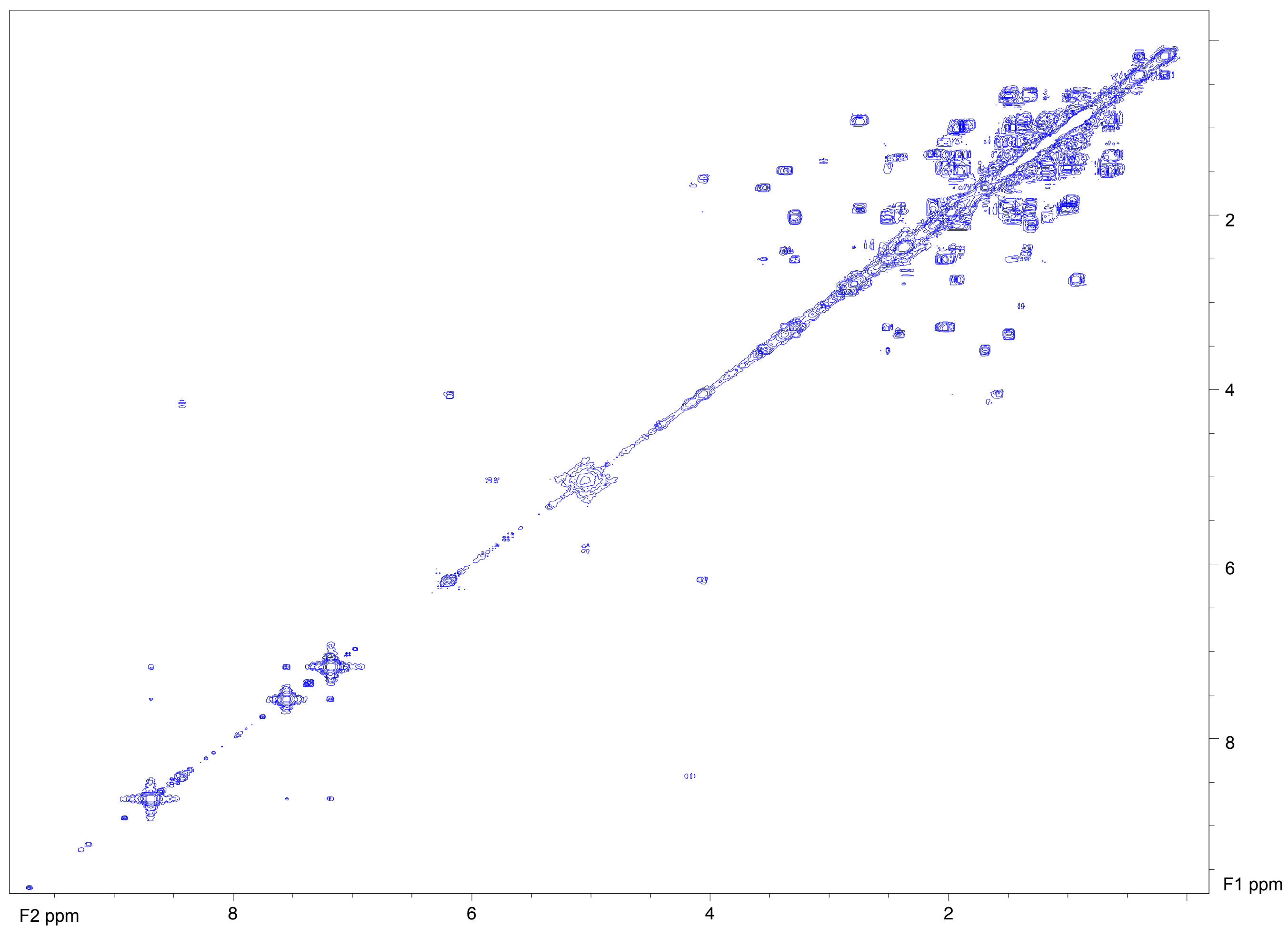
A23. HMBC spectrum of compound (**114**) in $CDCl_3$.



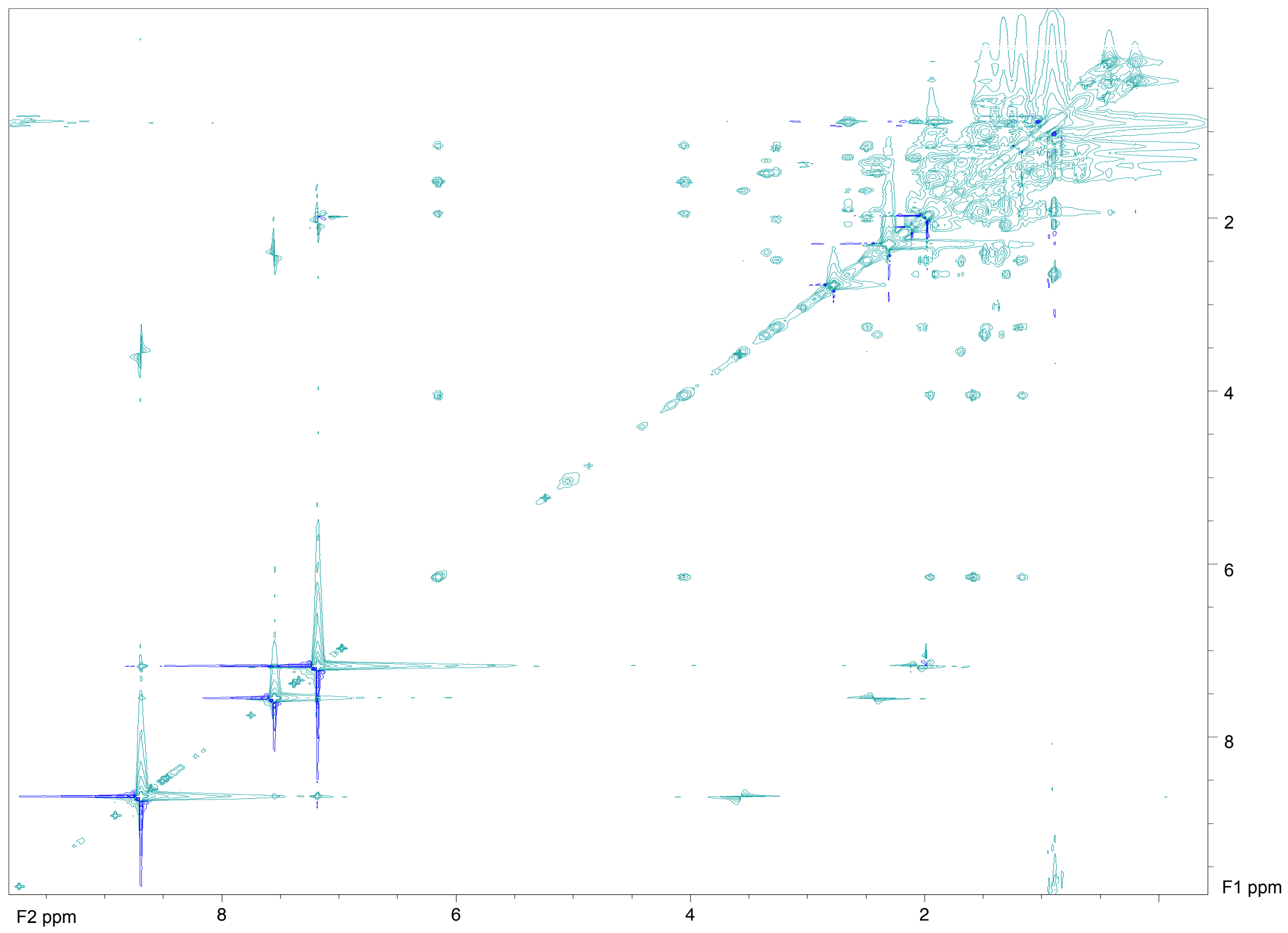
A24. NOESY spectrum of compound (**114**) in $CDCl_3$.



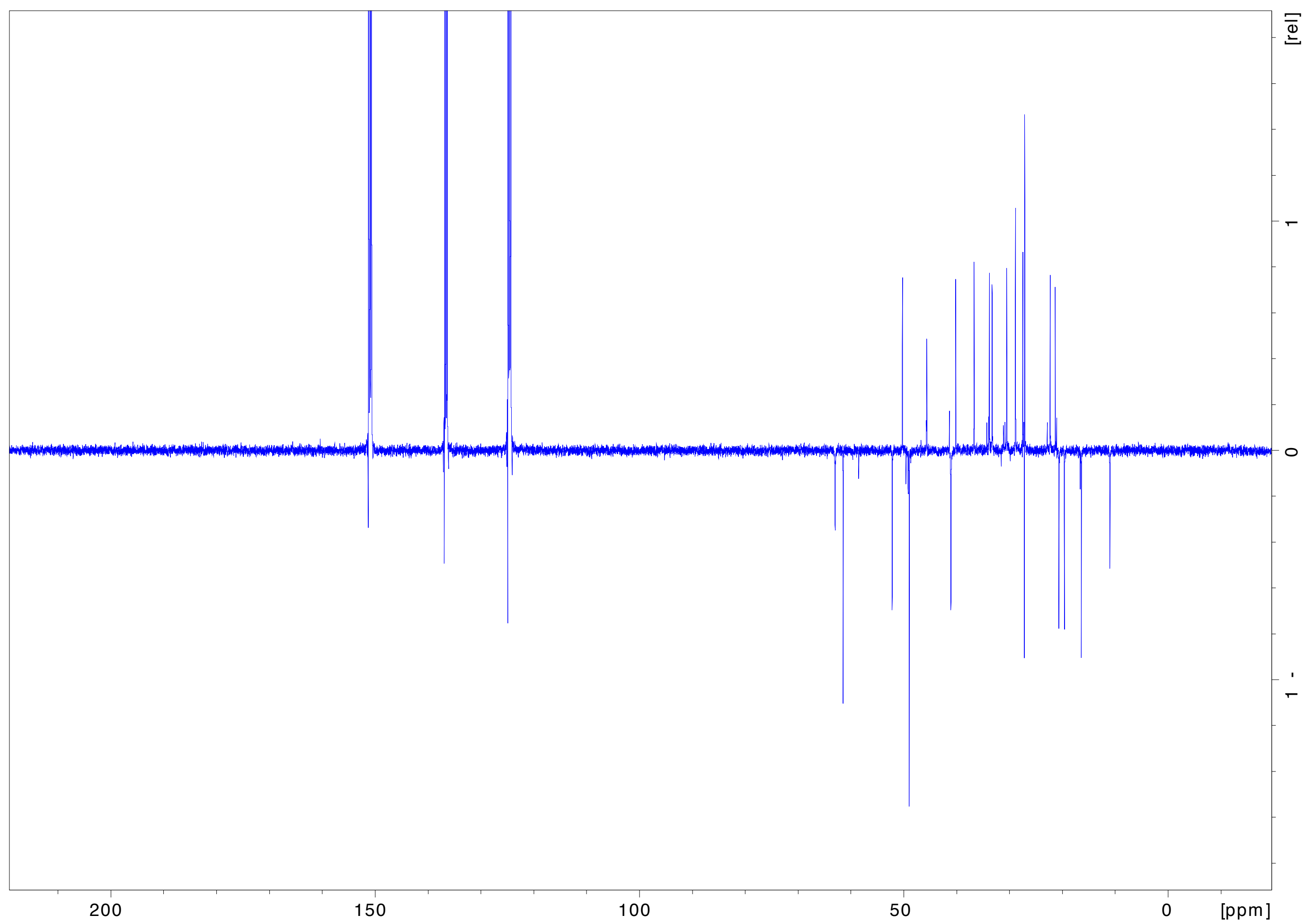
A25. ^{1}H-NMR spectrum of compound (**115**) in pyridine-d_5.



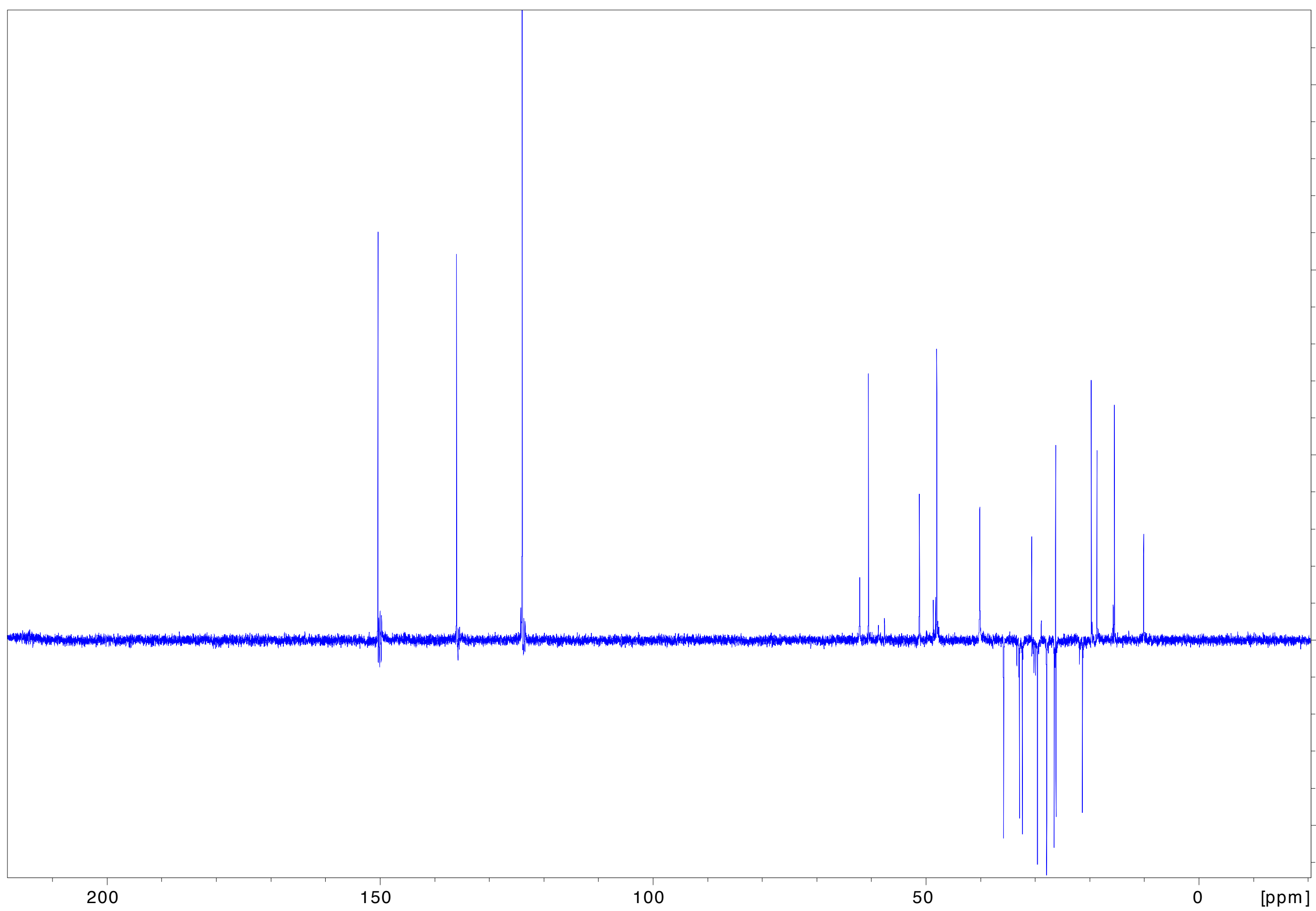
A26. COSY-45º spectrum of compound (**115**) in pyridine-d_5.



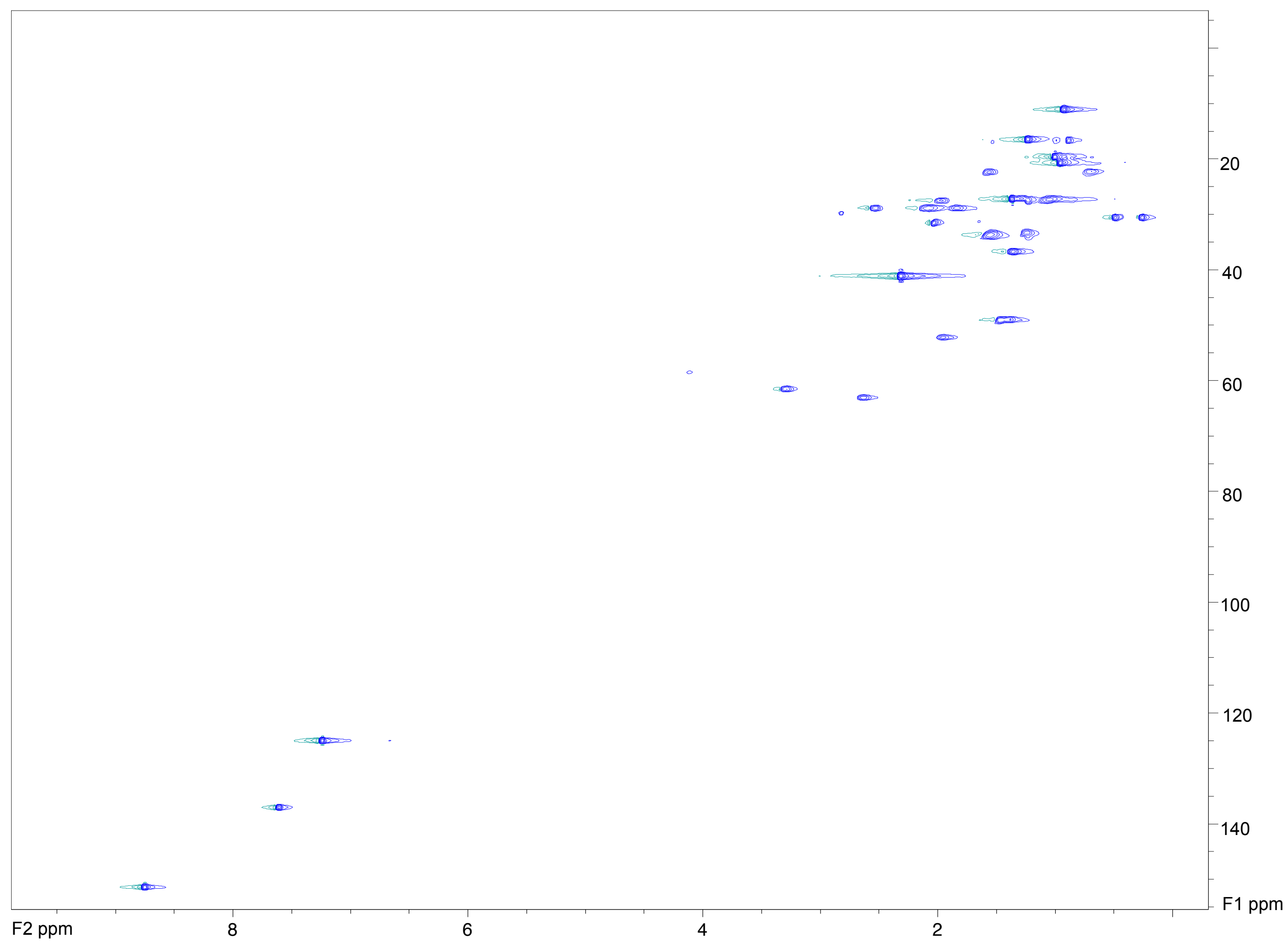
A27. TOCSY spectrum of compound (**115**) in pyridine-d_5 .



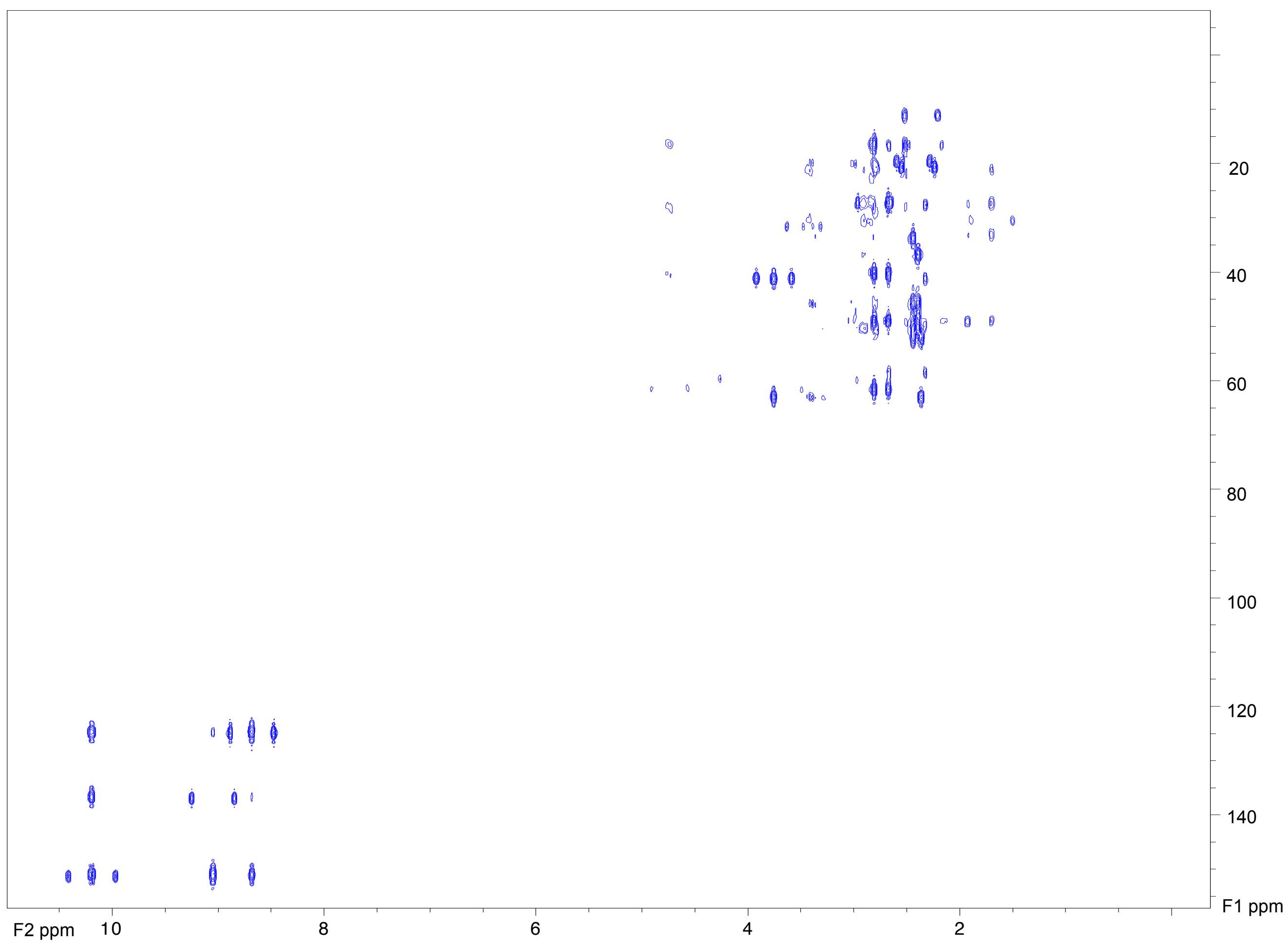
A28. APT spectrum of compound (**115**) in pyridine-d_5 .



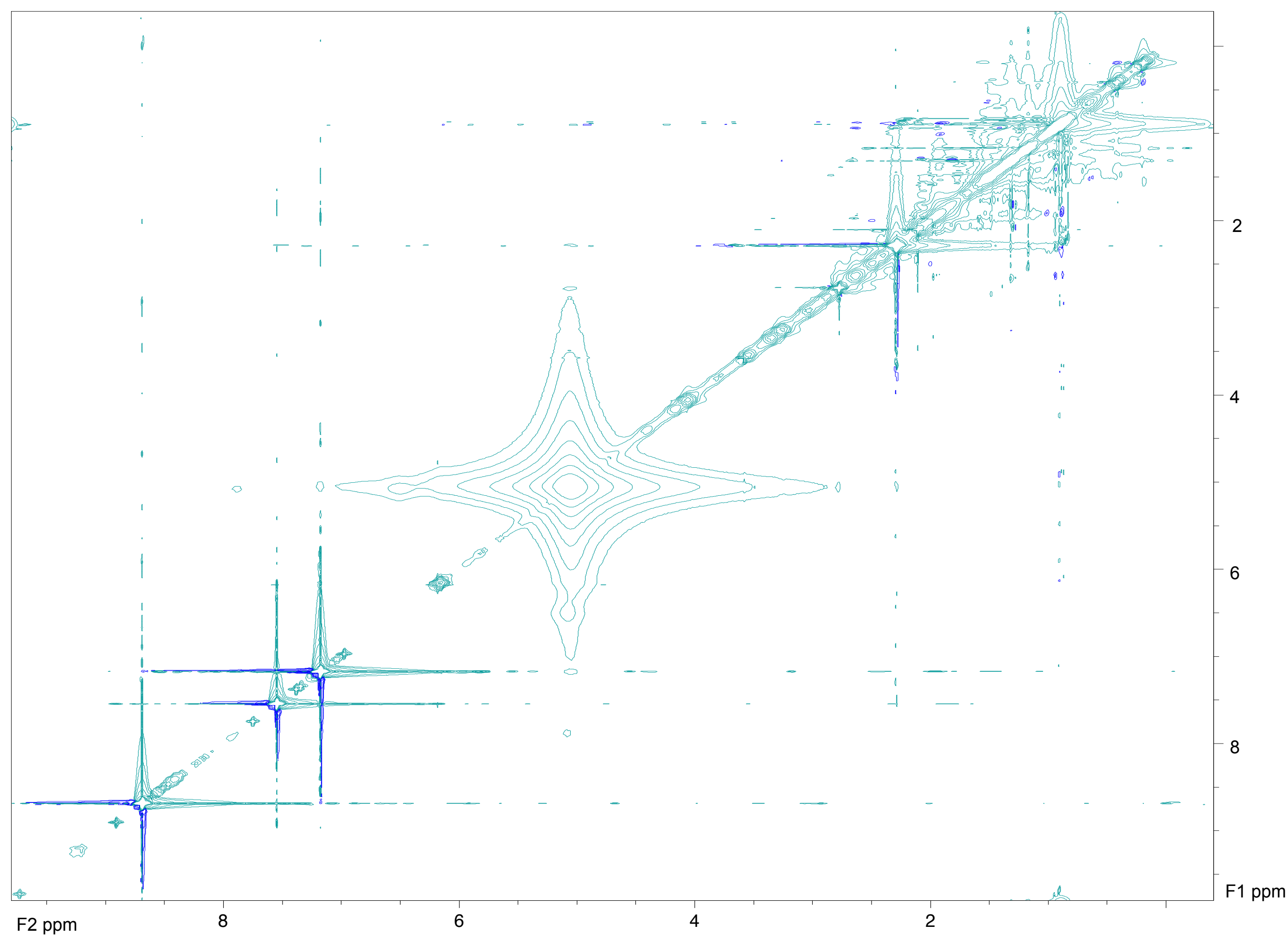
A29. DEPT-135 spectrum of compound (**115**) in pyridine-d_5 .



A30. HSQC spectrum of compound (**115**) in pyridine-d_5.



A31. HMBC spectrum of compound (**115**) in pyridine-d_5.



A32. NOESY spectrum of compound (**115**) in pyridine-d_5.