Terpenoids from the nudibranch *Phyllidia coelestis* Bergh

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I hereby certify that this work has not been accepted in substance for any degree, and is not being currently submitted in candidature for any degree.

..……. Signature

(Mr. Sunan Jaisamut)

Candidate

ชื่อวิทยานิพนธ์ สารประกอบเทอร์ปีนจากทากเปลือย *Phyllidia coelestis* Bergh
ผู้เขียน นายสุนันต์ ใจสมุทร
สาขาวิชา เภสัชศาสตร์
ปีการศึกษา 2556

บทคัดย่อ

จากการศึกษาองค์ประกอบทางเคมีของทากเปลือยชนิด *Phyllidia coelestis* Bergh จากเกาะห้า จังหวัดกระบี่ สามารถแยกสารประกอบ sesquiterpene ชนิด pupukeanane ที่มีหมู่ฟังก์ชันเป็นหมู่ formamide อยู่ในโครงสร้าง ซึ่งประกอบด้วยสารใหม่ 1 ชนิดที่มีลักษณะโครงสร้างเป็นแบบ tricyclo[4.4.0.0^{2,8}]decane คือ 1-formamido-10(1⟶2)-abeopupukeanane (**1**) และสารที่เคยมีรายงานมาแล้ว 1 ชนิดที่มีลักษณะโครงสร้างเป็นแบบ tricyclo[4.3.1.0^{3,7}]decane คือ 2-formamidopupukeanane (**2**) วิเคราะห์สูตรโครงสร้างโดยวิธีการทางสเปกโทรสโกปี ผลจากการทดสอบฤทธิ์ต้านเซลล์มะเร็ง HeLa (cervical), MCF-7 (breast), KB (oral cavity), และ HT-29 (colon) พบว่าสารทั้งสองชนิดมีฤทธิ์ต้านเซลล์มะเร็งปากมดลูก (HeLa) ในระดับดี และมีฤทธิ์ต้านเซลล์มะเร็งเต้านม (MCF-7) และมะเร็งช่องปาก (KB) ในระดับปานกลาง และมีค่า IC_{50} ในช่วง 0.05-10 μM.

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ABSTRACT

Chemical investigation of the nudibranch *Phyllidia coelestis* Bergh from Koh-Ha Islets, Krabi Province, Thailand, led to the isolation of two sesquiterpenes in the pupukeanane family that contain formamide group, including one new sesquiterpene with an unprecedented tricyclo[4.4.0.$0^{2,8}$]decane skeleton, named 1-formamido-10(1⟶2)-abeopupukeanane (**1**), and a known tricyclo[4.3.1.$0^{3,7}$]decane sesterterpene, 2-formamidopupukeanane (**2**). Both compounds were characterized on the basis of spectroscopic analyses. Antiproliferative activity was determined against HeLa (cervical), MCF-7 (breast), KB (oral cavity), and HT-29 (colon) cancer cell lines to show that both were active with IC_{50}s in a range of 0.05-10 μM.

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INTRODUCTION

1.1 General introduction

The chemical diversity of the marine natural products is immeasurable and therefore is an extraordinary resource for the discovery of new drugs. Marine organisms have the ability to produce chemical constituents with chemical foundations similar to those found in their terrestrial counterparts, but with an entirely different range in chemodiversity. For example, whereas terpenoid compounds are widely spread both in terrestrial and marine organisms - plants, animals, and microorganisms alike - a large number of marine-derived terpenes are unprecedented in terrestrial organisms but otherwise exclusive to marine origins. More than 20,000 marine natural products have been isolated from marine organisms (Hu *et al*., 2011). Several of those isolated compounds from marine sources have been reported to exhibit biological activities at the certain extent. Such impact undeniably indicates the potential of the oceans as a promising source of novel drugs.

Various classes of marine-derived bioactive compounds have been pursued towards medicinal applications, with a few prominent items have been already approved to be used clinically, and additional handful of compounds are now in their clinical trials. Whereas most of the marine natural products, including both the approved ones and those that are currently in clinical trials, are aimed toward anticancer chemotherapy, the emerging drug resistance encountered in the infectious diseases such as tuberculosis and malaria also contributes to the interest in the assessment of marine natural products towards drug-resistant microbes and parasites (Gerwick and Moore, 2012). Selected marine natural products, both commercially available and in different stages of clinical trials, are shown in Table 1. Their chemical structures are shown in Figure 1.

Table 1. The global marine pharmaceutical pipeline in 2012

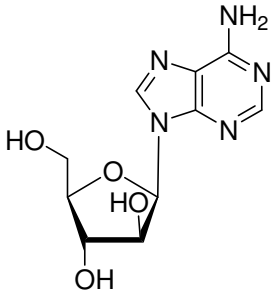
Compound name	Trademark	Source	Molecular target	Disease area
Approved				
cytarabine (ara-C)	Cytosar-U®	*Criptotethya crypta* (sponge; synthetic derivative of spongouridine)	DNA polymerase	leukaemia
vidarabine (ara-A)	Vira-A®	*Criptotethya crypta* (sponge; synthetic derivative of spongouridine)	viral DNA polymerase I	antiviral
ziconotide	Prialt®	*Conus magus* (cone snail)	N-type Ca channel	pain
eribulin mesylate (E7389)	Halaven®	*Halichondria okadai* (sponge; synthetic derivative of halichondrin B)	microtubules	metastatic breast cancer
ω-3-acid ethyl esters	Lovaza®	fish; synthetic derivative of ω-3 fatty acids	triglyceride synthesizing	hypertriglyceridemia
trabectedin (ET-743) (EU registered only)	Yondelis®	*Ecteinascidia turbinata*(tunicate)	minor groove of DNA	ovarian cancer
brentuximabvedotin (SGN-35)	Adcetris®	*Dolabella auricularia* (mullusk; synthetic derivative of dolastatin10)	CD30 and microtubules	Hodgkin lymphoma

Table 1. (cont.)

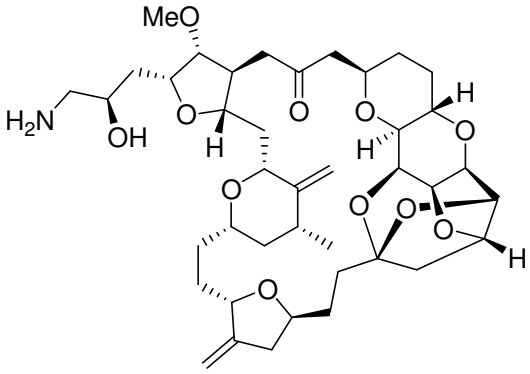
Compound name	Trademark	Source	Molecular target	Disease area
Phase III				
plitidepsin	Aplidin®	*Aplidium albicans* (tunicate)	Rac1 and JNK activation	acute lymphoblastic leukemia
Phase II				
DMXBA (GTS-21)	NA	*Amphiporus lactifloreus* (nemertines; synthetic derivative of anabaseine)	α7 nicotinic acetylcholine receptor	cognition, schizophrenia
plinabulin (NPI 2358)	NA	*Aspergillus ustus* (marine fungus; synthetic analogue of phenylahistin)	microtubules and JNK stress protein	non-small cell lung cancer
elisidepsin	Irvalec®	*Aplidium albicans* (marine mollusk; synthetic derivative of kahalalides)	plasma membrane fluidity	non-small cell lung cancer
PM00104	Zalypsis®	*Jorunna funebris* (nudibranch; synthetic derivative of jorumycin)	DNA binding	cervical cancer
glembatumumabvedotin (CDX-011)	NA	*Dolabella auricularia* (mullusk; synthetic derivative of dolastatin10)	glycoprotein NMB & microtubules	breast cancer

Table 1. (cont.)

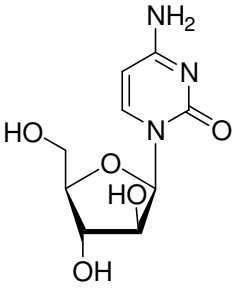
Compound name	Trademark	Source	Molecular target	Disease area
Phase I				
marizomib (salinosporamide A; NPI-0052)	NA	*Salinospora tropica* (marine bacteria)	20S proteasome	cancer (proteasome inhibitor)
lurbinectedin (PM01183)	NA	*Ecteinascidia turbinata* (tunicate; synthetic derivative of trabectedin)	minor groove of DNA, nucleotide excision repair	ovarian cancer
SGN-75	NA	*Dolabella auricularia* (mullusk; synthetic derivative of dolastatin10)	CD70 and microtubules	kidney cancer
hemiasterlin derivative (E7974)	NA	*Hemiasterella minor* (sponge)	microtubules	colorectal cancer
bryostatin 1	NA	*Bugula neritina* (bryozoan)	protein kinase C	colorectal cancer, Alzheimer's
pseudopterosins	NA	*Pseudopterogorgia elisabethae* (soft coral)	eicosanoid metabolism	wound healing



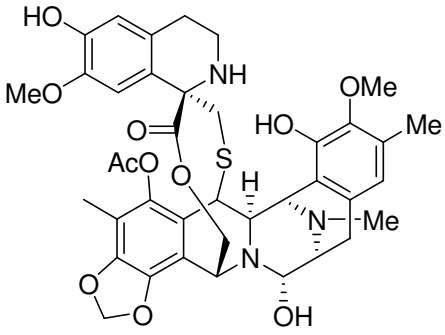
Vidarabine (Ara-A)



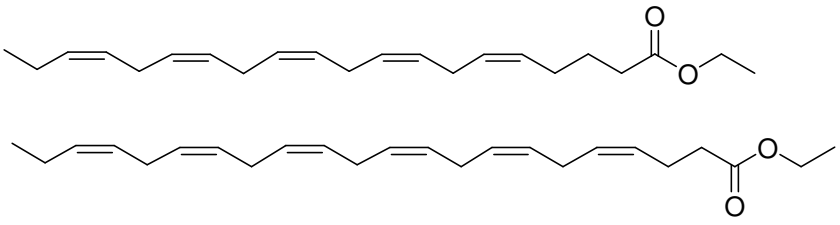
Eribulin Mesylate (E7389)



Cytarabine (Ara-C)

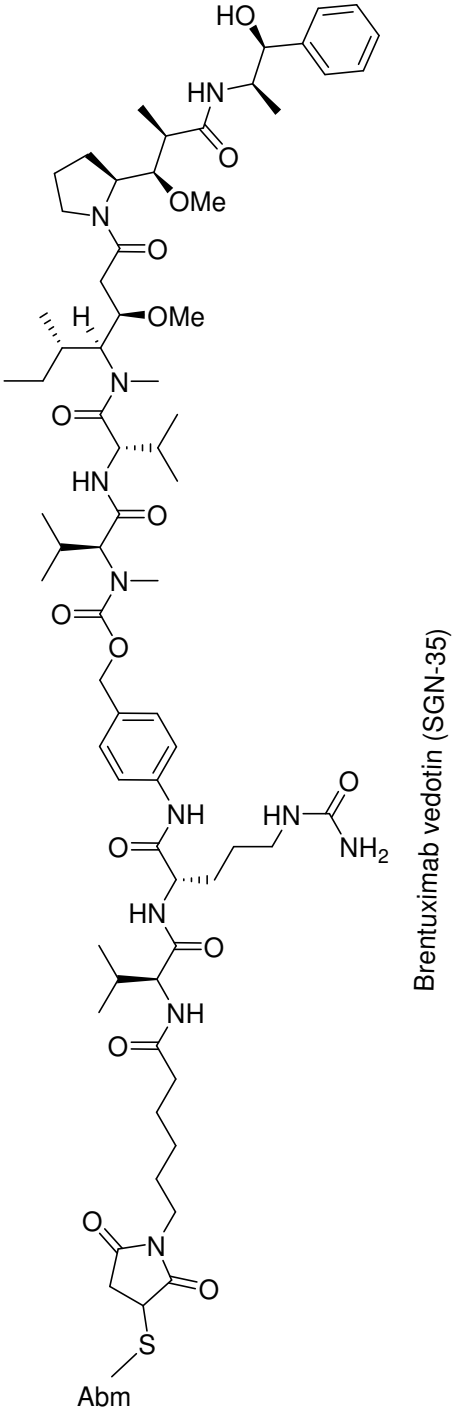


Trabectedin (ET-743)

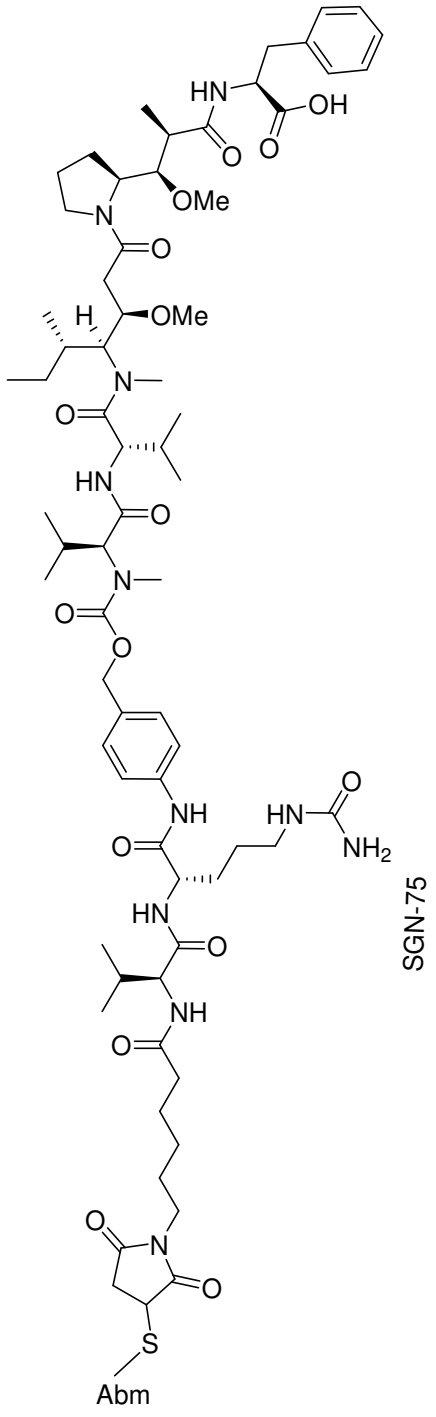


ω-3-acid ethyl esters

Figure 1. Approved marine-derived drugs and analogues in clinical trials.

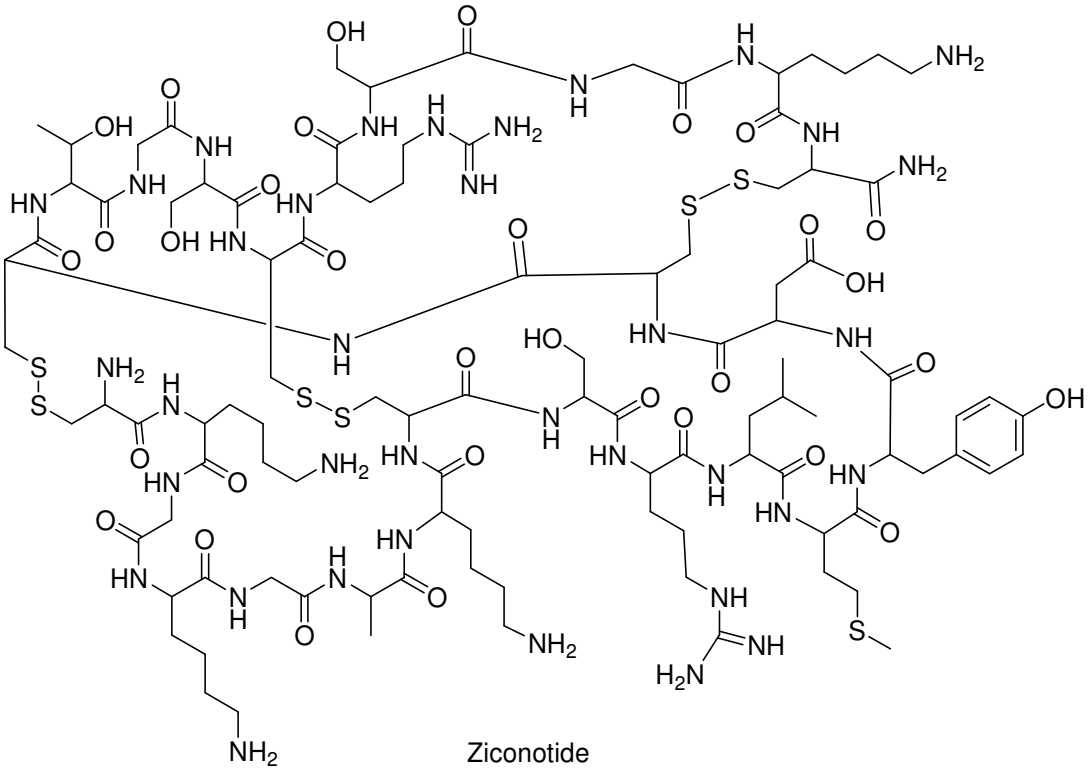


Brentuximab vedotin (SGN-35)

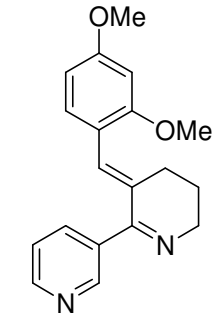


SGN-75

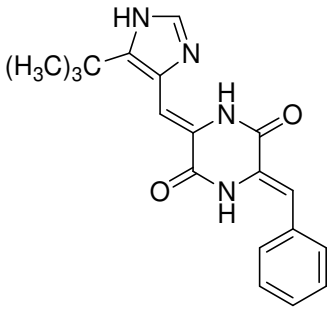
Figure 1. (cont.)



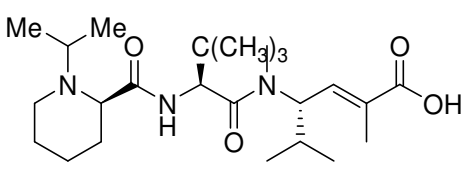
Ziconotide



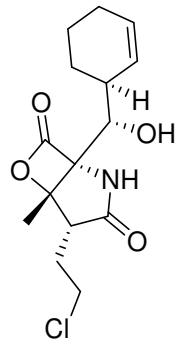
DMXBA (GTS-21)



Plinabulin (NPI 2358)

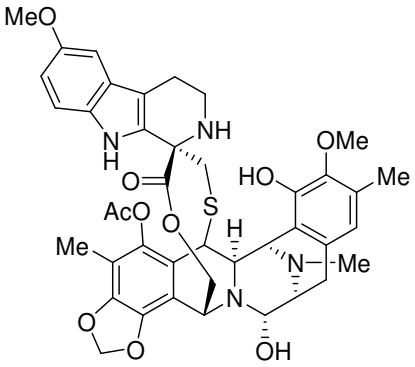


E7974

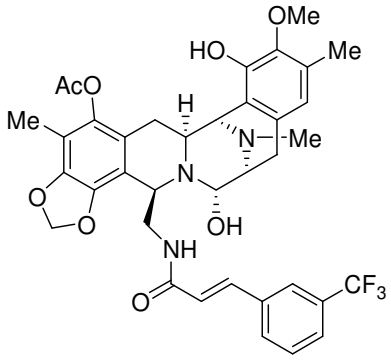


Marizomib (Salinosporamide A; NPI-0052)

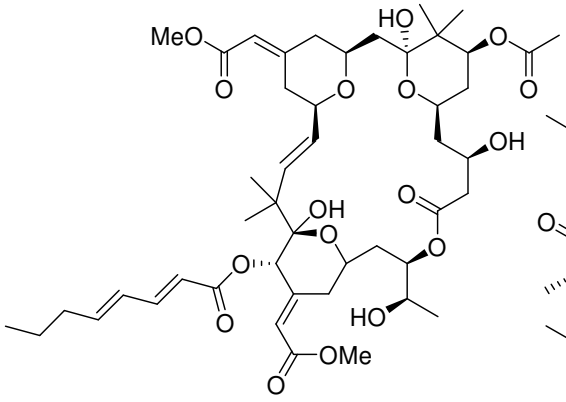
Figure 1. (cont.)



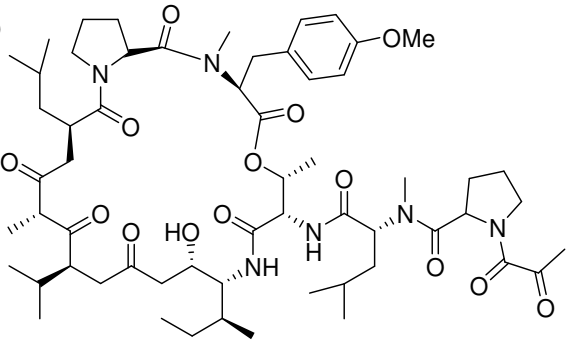
PM01183



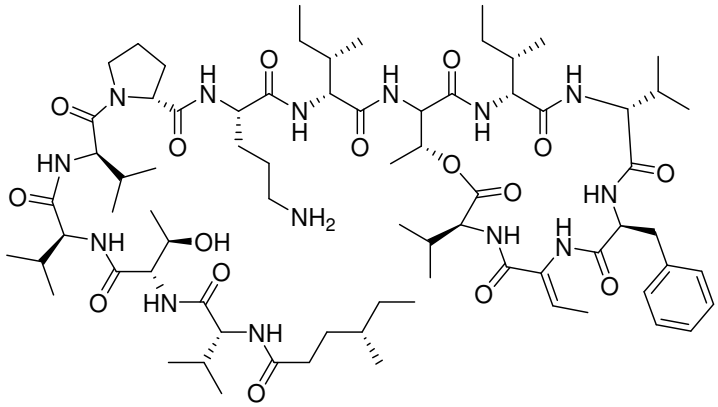
PM00104 (Zalypsis)



Bryostatin 1



Plitidepsin



Elisidepsin

Figure 1. (cont.)

1.2 Terpenoid derivatives

Terpenoid compounds are a large class of organic compounds that consist of isoprene, or isopentenyl, units stringing altogether in a so-called head-to-tail manner. Natural sources of terpenoid compounds vary widely from plants, to animals and microorganisms. Due to the building block of isoprene units, terpenoids can be categorized simply based on the number of isoprene units used as the immediate precursors to construct their chemical structure, ranging from hemiterpenes (C5), monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), sesterterpenes (C25), triterpenes (C30), and polyterpenes (>C30). Upon connection of all the acquired isoprene units, the resulting multiple isoprene intermediates undergo a series of modification, including cyclization, aromatization, and functionalization, particularly with oxygenated functional groups. Also, very often, the carbocation intermediates shunt their ways toward a series of rearrangements. Such modifications make terpenoid compounds among the most structurally diversified classes of natural products.

1.2.1 Structural variation

As mentioned above, terpenoid compounds are synthesized biogenetically through the connecting isoprene units, hence categorizable by the number of the isoprene units participating in such biosynthetic processes. It is also possible to classify terpenoid compounds by means of the structural complexity; i.e., how the intermediates resolve themselves during cyclization and transformation.

1.2.1.1 Linear terpenes

Bearing the simplest non-cyclized structures, linear, or aliphatic, terpenoid compounds are basically the precursors and intermediates of other structurally advanced and highly functionalized cyclic terpenoids. Linear terpenoid compounds that are readily presented as natural products in plants and other producing organisms are also available, although very limited

variations. The sizes of the compounds ranged from hemiterpenes and the most commonly found monoterpenes to compounds as large as tetraterpenes. Funtionalization mainly involves oxygenation as in isovaleric acid (from *Valeriana pavonii*, Giraldo *et al*., 2010), and also in a wide range of volatile components as in linalool, (for example, as a component in camphor oil from *Cinnamomum camphora,* Suga *et al*., 1972). Tetraprenes, or tetraterpenes, and related carotenoids are also among the most commonly presented in natural sources. As carotenoid pigments, tetraterpenes, for example, lycopene from tomato (*Lycopersicon esculentum* Miller), can be widely found in yellow and red fruits and vegetables. Another group of linear terpenoids that are widely spread through plant kingdom is phytol, which in fact is one composition in chlorophylls.

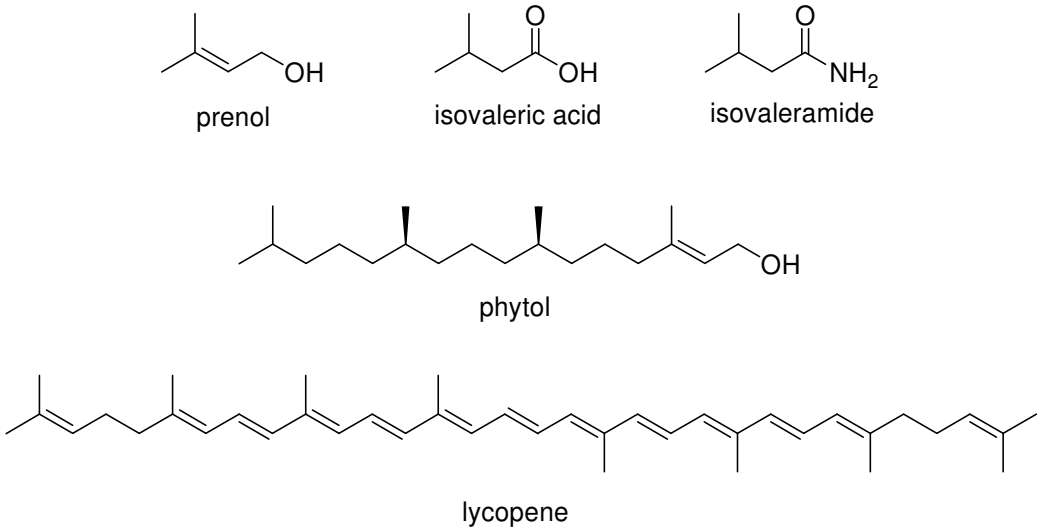


Figure 2. Linear terpenoid compounds

1.2.1.2 Carbocyclic terpenoid compounds

As mentioned earlier, most terpenoid compounds, upon the connection of the acquired isoprene units into a linear terpene intermediates, undergo cyclization steps to furnish the structures toward the chemical structures of carbocyclic terpenoid compounds as commonly found in natural products. Hence, more than 95% of terpenoid compounds possess cyclic structures to a certain extents, ranging from one-ring or monocyclic ring system, to multiple cyclic structures. Such cyclic structures may or may not retain parts of the pre-existing linear

structures as their side chain unit. Also, although the structure of thermodynamically stable six-membered ring are the most frequently encountered, other ring size, either as small as three-, four-, and five-membered rings, or as large as 10-carbon macrocyclic, or even larger, can be found. Oxygenation, olefiniation, and aromatization are the most common chemical modification, although other functionalizations such as nitrogenation are also frequently reported. The sizes of the terpenoid structures also range widely and are covered in almost every class of terpenoid compounds, from monoterpenes, to sesqui-, di-, sester-, tri- and tetraterpenes.

Shown below are some examples of carbocyclic structures of terpenoid compounds. As stated above, the structures of carbocyclic terpenoid compounds range widely from the monocyclic monoterpenes, which are possibly the most common, and most widely found as the primary compositions in the fragrant that provide a specific and characteristic scent of each particular plants. For example, (+)-carvone is the primary components in the scent of caraway, and also found in several other flowers and spices including dill seed oil (*Anethum graveolens*) and caraway seeds (*Carum carvi*) (Sell, 2003). Its enantiomer, (-)-carvone, on the other hand, is the primary component that yields the scent of mint, and also found in spearmint oil (*Mentha spicata*) (Sell 2003). Other examples of carbocyclic terpenoids in essential oils may include terpinene, thymol, and carvacrol (Hyldgaard *et al*., 2012)

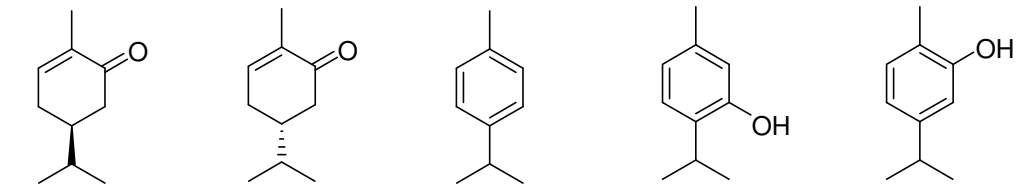


Figure 3. Carbocyclic terpenoid compounds in essential oils

Apart from essential oils, which constitute the major class of carbocyclic terpenoid compounds, the following examples are terpenoids presented in various sizes and found in various sources, ranging from sesquiterpenes, such as lyophyllone A (from the mushroom *Lyophyllum transforme*; Clericuzio *et al.*, 2013), diterpenes, such as mallonicusin A (from stems

of *Mallotus japonicus*; Li *et al.*, 2013), sesterterpenes, such as 12-*O*-deacetylscalarafuran (from a marine sponge *Spongia* sp; Tsukamoto *et al.*, 2003), triterpenes, such as 21-ketobetulinic acid (from *Machaerocereus eruca*; Ye *et al.*, 1998), and tetraterpenes, such as astaxanthin (from the shrimp *Metapenaeus dobsoni*.; Ushakumari and Ramanujan, 2013).

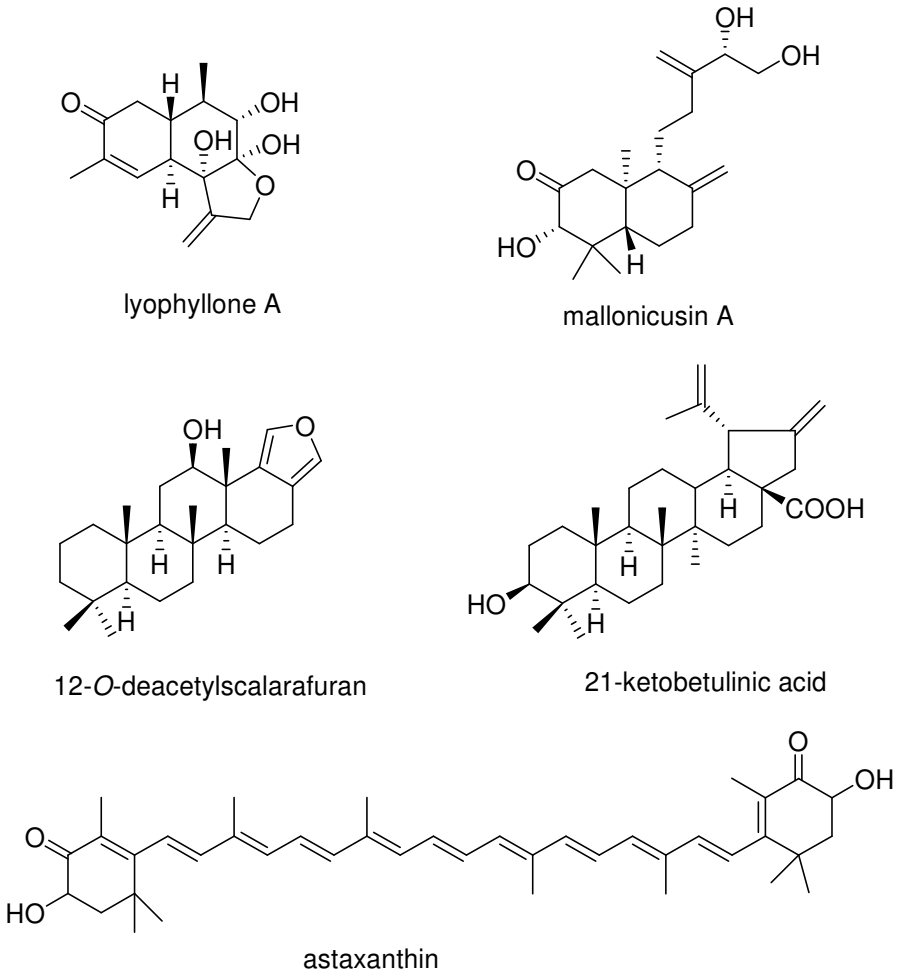


Figure 4. Carbocyclic sesqui-, di-, sester-, tri- and tetraterpenes

1.2.1.3 Rearranged terpenoid compounds

As described above that terpenoid compounds are originally biosynthesized through the connection of a series of acquired isoprene units, which naturally presented as either dimethylallyl pyrophosphate or as isopentenyl pyrophosphate. The connection involves mainly addition of isopentenyl carbocation onto pyrophosphate - activated allyl nucleophiles, resulting in

a carbocation that may undergo a cascade of cyclization or functionalization. With the carbocation as a common intermediate, rearrangement, particularly through either methyl, hydride, or alkyl shifts, may also take place. On the other hand, the olefination that normally furnish the cyclization, and the oxygenation, which is the most common functionalization for terpenoid compounds, may be followed by a series of oxidative cleavage, leading to ring expansion/rearrangement (as in Baeyer-Viliger oxidation), or loss of certain part in the carbon skeleton.

The most subtle form of rearrangement among terpenoid compounds in fact can be seen in steroids and triterpenoid. All the steroid derivatives retain only two axial methyl groups on their C-10 and C-13, whereas three other methyls lost during the cyclization steps. The oxidative cleavages on the side chains also yield steroid structures of different classes. Cholesterol, for example, has 27-carbon skeleton, of which only three methyls were cleaved. Estradiol, on the other hand, has only 18 carbons on its skeleton, losing 10 carbons on the side chain upon the modification towards the final structure.

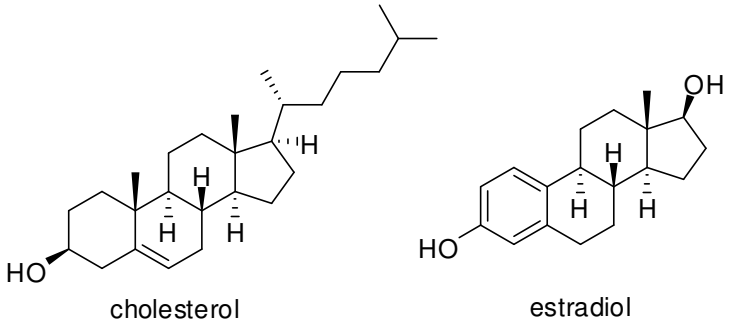


Figure 5. Cholesterol and estradiol

Several other rearranged terpenoids that involve either carbon loss and/or alkyl/hydride shifts are very well known. Some well-deserved examples include ginkgolides (from *Ginkgo biloba*; Jaracz *et al.,* 2004) and limonoids (from the seeds of *Azadirachta indica*; Kikuchi *et al.,* 2011, seeds of *Lansium domesticum* Corr.; Saewan *et al.,* 2006, *Harrisonia abyssinica*; Rugutt *et al.,* 2001), both of which constitute a major classes of natural products that have been widely known and used in pharmaceutical and agricultural industries.

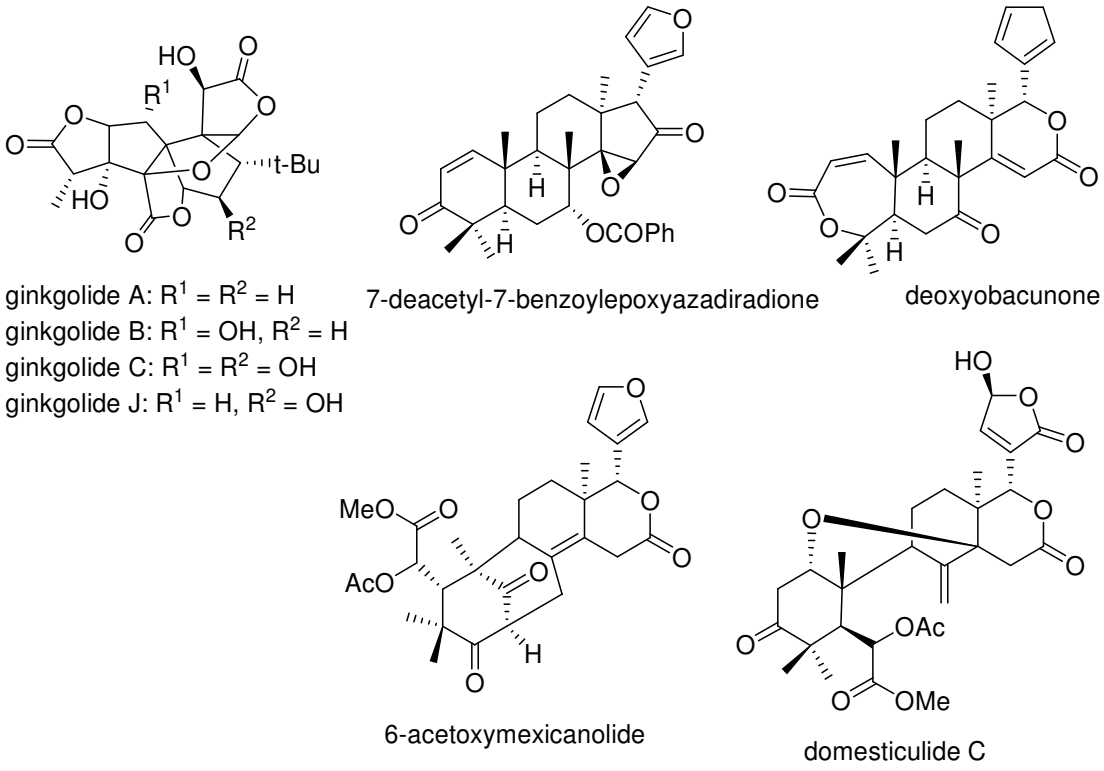


Figure 6. Ginkgolides and limonoids

1.2.2 Roles terpenoid compounds in plants and producing organisms

Several terpenoid compounds have been extensively studied for their functions in the producing organism. Several terpenoid compounds have been found to play vital roles in plants and animals that produce such compounds. In certain specific cases, it was also found that some animals can also exploit certain terpenoids by sequestering such compounds from the producing plants, mainly through dietary accumulation, and use such compounds in their benefits.

The most common role of terpenoids in producing plants is to attract animals and insects that may act as pollen carriers, particularly well exemplified by volatile oils from most flowers. Apart from the pleasant scents, volatile oils are primary signals spreading airborne as the primary messages to the pollinating insects, birds, or even bats, that the flowers are blooming, and the pollens are ready to be transferred. In certain cases, the volatile oils are produced in such a

specific composition, and are intended to attract only specific pollinating species. The volatile oils that constitute the smell of fruits is also made for a similar propose of attracting the animals that feed on such ripening fruits therefore help spreading the seeds.

On the other hand, several volatile oils are founds accumulating in other parts of plants, i.e., leaves, barks, woods, and rhizomes. Very often, volatile oils found in these parts, despite pleasant to human, are rather pungent, and are used to protect the plants as a repellant to chase away pests and herbivorous animals. Good examples can be seen in most plants in the family Lamiaceae, such as mints and basils, in which the volatile oils are accumulated in glandular trichomes thoroughly covering leaves and stems. The repelling effects from the Lamiacious volatile oils chase away both the herbivorous animals and also prevent most insects from laying their eggs on the leaves and stems. The repelling effects of volatile oils are in fact appreciated widely since the ancient time, and volatile oils from several plants, such as citronella oil from *Cymbopogon nardus* and volatile oils from several citrus peels, have long been used as mosquito repellants.

In addition, volatile oils also have a very strong antiseptic effect, and very well known to provide a primary protection to plants against invading pathogenic bacteria and fungi. Such antiseptic properties of volatile oils are also very well known. The culinary uses of herbs and spices found in every culture are not only for the seasoning purposes, but also as the preservatives to prevent food spoiling. Several herbs that contain volatile oils are also used as an antiseptic in wound dressing.

Other classes of terpenoid compounds are also well known to provide the repelling effects to the producing plants, particularly the compounds that have deterring taste. The examples include modified triterpenes in quassinoid and limonoid families. All of the triterpenes in these two classes are unpleasantly bitter, hence repelling the herbivorous animals within the very first nib from their leaves, and also conveying the message that not only the plants are unpleasantly not edible, but they also are rather too poisonous to be fed on.

In addition to the unpleasant taste, several terpenoid compounds provide protective effect through the toxicity. For examples, quassinoids and limonoids are also among a

wide range of terpenoid compounds that have toxic effects. Limonoids, and more specifically nimbolides, have an antifeedant effect in the insects that cause the insects to loss all their appetite. Neem oil, Neem oil from *Azadirachta indica*, for example, has been used as an insecticide in the organic farms. Several toxic terpenoid compounds are also well known; for examples, picrotoxin from *Anamirta cocculus*, and aconitine, terpene alkaloid from *Aconitum napellus*, are among the most toxic compounds found in nature, and are very well speculate to provide a protective benefit to their producing plants. The protective effects of toxic terpenoids do not limit in the producing organisms. Certain animals are also known to immune from certain toxic terpenoids, and can even sequester such toxic components, through the dietary accumulation, and use such compounds to their own benefit. Some example include the accumulation of cardiac glycosides in the caterpillars of monarch butterfly *Danaus plexippus*, which feed on the leaves of *Calotropis gigantea*, and use such toxic glycosides as their chemical defense. Also very well-known are the accumulation of toxic terpenoids in several nudibranchs, which are the predators of terpenoid-producing sponges, and collect such toxic compounds to be used as their own chemical weapons. For examples, the nudibranch *Phyllidia pustulosa* are known to accumulate 2-isocyanopupukea-nane, the toxic terpenoids found in the sponge *Halichondria* cf. *lendenfeldi* (Kassuahlke *et al*., 1991).

Terpenoids also play an important role of hormones. Apart from steroidal hormones, which are widely found in every animal phylum, certain other classes of terpenoid compounds have also been used as plant hormones. The most well-known group of hormonal terpenoids are giberrelins, which provide growth effects in plants.

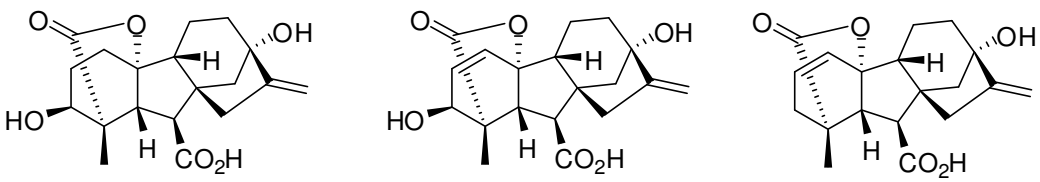


Figure 7. Giberrelins

1.2.3 Application of terpenoid compounds

Several terpenoid compounds possess pharmacological properties and have been widely used both in traditional and modern medicines. Spices, in which the terpenoid-derived volatile oils constitute the major components, have been long used in culinary and perfumery industries. The antiseptic properties of the terpenoids in volatile oils, as described previously, are also well recognized, and several antiseptic products currently used are based on the volatile oils. Some very well-known examples of other currently and clinically used terpenoids include digoxin, a cardiac glycoside from *Digitalis lanata*, which is used in congestive heart failure; paclitaxel, the anticancer from *Taxus brevifolia* used in the treatment of breast cancer; and artemisinin, the antimalarial drug from *Artemisia annua* (Klayman *et al*., 1984). Also, a number of medicinal herbs widely used in alternative medicines contain terpenoid compounds as the active ingredients. These include ginkgo leaves (*Ginkgo biloba*), in which ginkgolides constitute the famous effect of memory repair; ganoderma or reishi mushrooms (*Ganoderma lucidum*), which contain the triterpenes of lanostane derivatives (Cheng *et al*., 2012; Wu *et al*., 2001), and ginseng (*Panax ginseng*), one of the most widely used medicinal herbs, in which ginsenosides (Kim, 2012) are responsible to the famous adaptogenic effect.

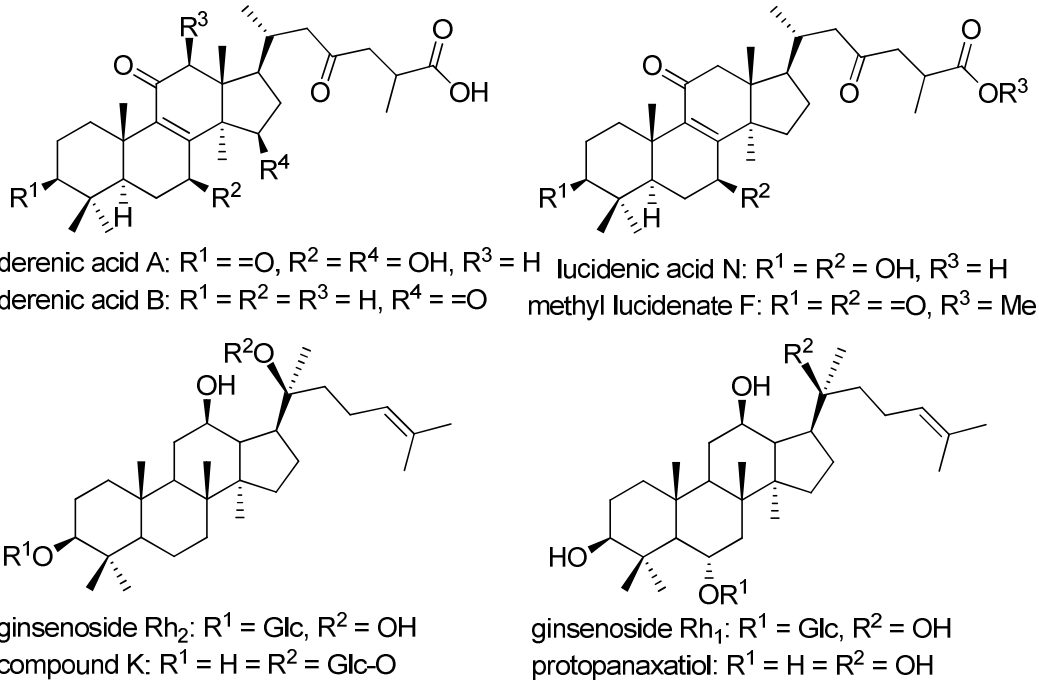


Figure 8. Triterpenes lanostane derivatives and triterpene glycosides

In addition to the currently used medicines and herbal products, a wide range of terpenoids from plants and other natural sources have been reported to possess strong and promising biological activities, some of which also have been proposed to exhibit their activities with unprecedented modes of actions. Some examples may include maslinic acid from olives, which has been reported to inhibits serine proteases, key enzymes necessary for the spread of HIV within an individual's body and antiproliferative effects on colon cancer cells (Juan *et al*., 2008), and globulol from fruits *Eucalyptus globulus* Labill (Myrtaceae), which was reported with an antimicrobial activity (Tan *et al.*, 2008).

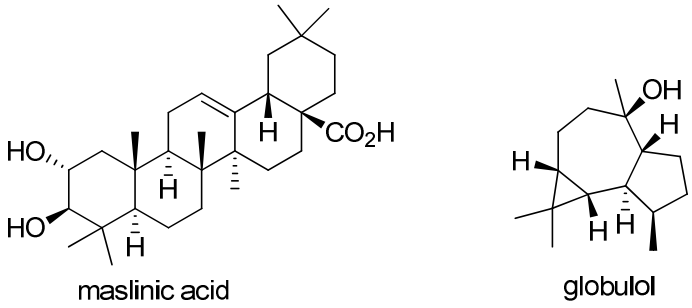


Figure 9. Maslinic acid and globulol

1.3 The pupukeanane-type sesquiterpenes

Pupukeanane-type sesquiterpenes, which constitute the focal point of this dissertation, are a very small family of terpenoid compounds. To date, less than 20 derivatives, rearranged analogs included, have been reported. All the pupukeanane derivatives are of exclusively marine origins. The first member of the pupukeanane family, 9-isocyanopupukeanane, was reported from the nudibranch *Phyllidia varicosa* (Burreson *et al*., 1975). The compound was postulated to be sequestered by the nudibranch through its diet. The postulation was later proved as the compound was isolated from the sponge *Hymeniacidon* sp. (Hagadone *et al*., 1979), on which the nudibranchs were found grazing. It has been proposed that the nudibranchs sequester such toxic terpenoids from their sponge prey, accumulate and employ the compounds as their chemical weapon, secreting into their mucus to fend off their predators, or sometime even to prey on other animals (Okino *et al*., 1996).

Chemically, the pupukeanane sesquiterpenes possess the unique tricyclo [4.3.1.$0^{3,7}$]decane, or isotwistane carbon framework (Figure 10) with two additional methyl and

one isopropyl groups substituted on C-1, C-6, and C-4, respectively. Of special interest is the isopropyl moiety, which resides on the unfavorable *endo* position (Simpson and Garson, 2004), and reflect the specific cyclization patterns from the proposed amorphene precursor (see below). Most of the pupukeanane derivatives are functionalized by either the rare isonitrile or related functionalities, including isothiocyanate, isocyanate, and formamide groups. The positions substituted by such groups locate very often, but not exclusively, on C-2 and C-9 (Hayes *et al*., 1975; Patil *et al*., 1997).

Rearrangement within the bridged tricyclic structures of the pupukeananes are very common, and at least two other closely related arranged derivatives have been reported, namely the neopupukeananes and allopupukeananes. Neopupukeananes is characterized by a 9-isopropyl-3,6-dimethyltricyclo[4.3.1.$0^{3,7}$]decane carbon framework. Similar to the pupukeananes, the neopupukeananes have also been associated with isonitrile-related functional groups. The prototype of the neopupukeananes can be exemplified by 2- thiocyanatoneopupukeanane, first

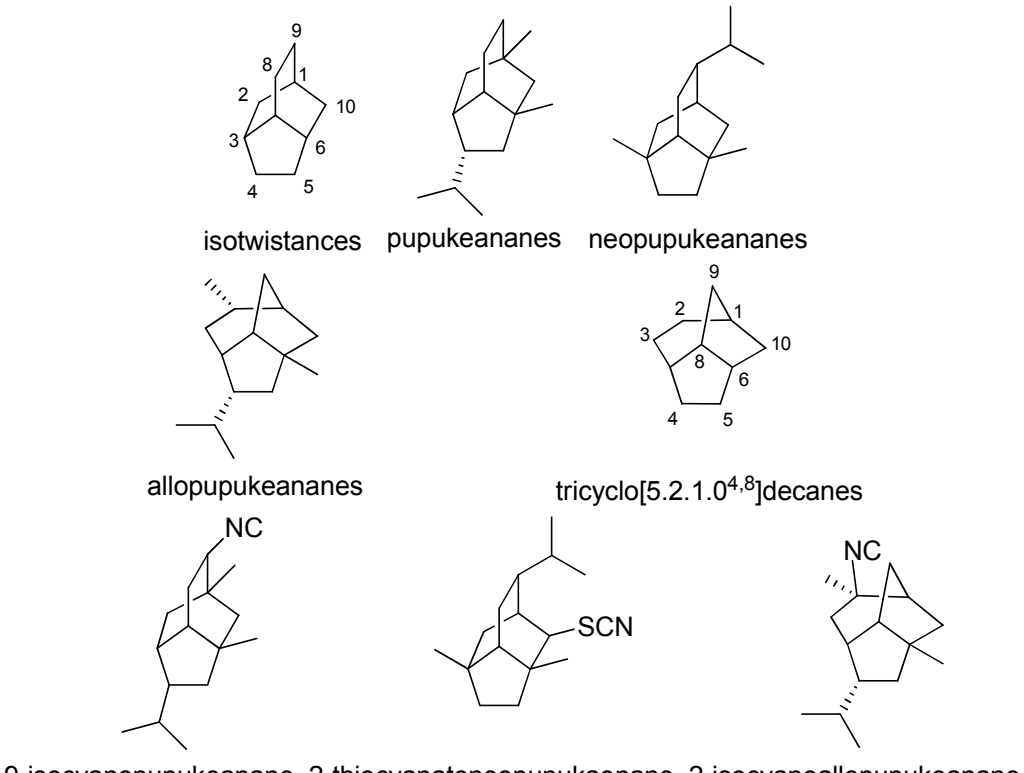
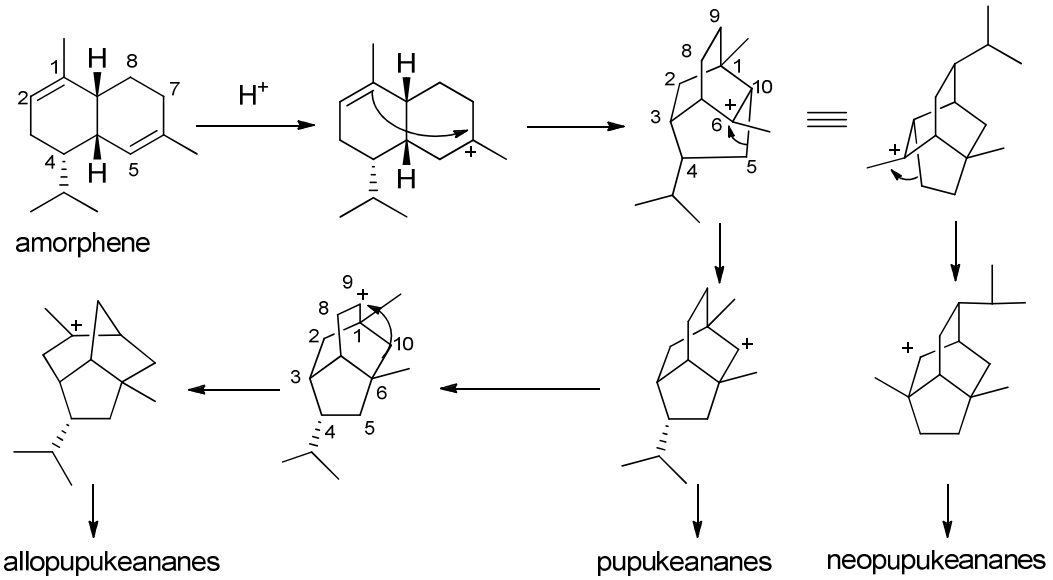


Figure 10. Structures of isotwistanes, tricyclo[5.2.1.$0^{4,8}$]decanes, pupukeananes, neopupukeananes and allopupukeananes.

isolated from the sponge *Phycopsis terpnis* (Pham *et al*., 1991). Another group of rearranged derivatives, the allopupukeananes, possesses a tricyclo[5.2.1.$0^{4,8}$] decane carbon framework. An isonitrile derivative, 2-isocyanoallopupukea-nane, was first isolate from the nudibranch, *Phyllidia pustulosa* (Fusetani *et al*., 1991).

Biogenetically, all the pupukeanane-type sesquiterpenes have been proposed to share a common biogenetic origin (Scheme 1; Dumdei *et al*., 1997). Having amorphene as an immediate precursor, 1,6-cyclization transforms the amorphenium cation into a bridged tricyclic intermediate, which undergoes the second rearrangement either through a 5(10→6) shift toward the pupukeananes or through an 8(5→6) shift toward the neopupukeanane. An additional 10(1→9) shift on the resulting pupukeanane cation yields the allopupukeananes (Srikrishna *et al*., 2006). Notice here that, as mentioned above, the configuration of the pupukeanane skeleton is in fact governed by the configuration of amorphene precursor, in which *cis*-configuration of the amorphene ring system dictate the isopropyl side chain to reside on the least favored *endo* side.



Note; Atom numbering of the first transformation is referred to the structure of amorphene, whereas the following transformations were to that of pupukeanane.

Scheme 1. Biogenetic pathway of pupukeananes, neopupukeananes and allopupukeananes.

Being a relatively small group of bridged sesquiterpenes, to date, there have been only 13 naturally occurring pupukeananes and related sesquiterpenes isolated. The list of the pupukeananes and rearranged derivatives is shown in Table 2.

Table 2. Biological sources and activities of pupukeanane-type sesquiterpenes

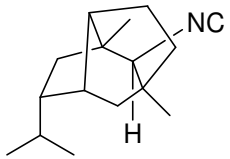
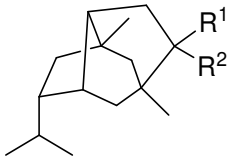
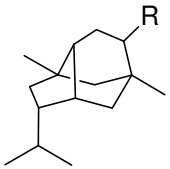
Structures	Sources	Biological activities	References
 2-isocyanopupukeanane	*Phyllidia varicosa* (nudibranch) and *Hymeniacidon* sp. (sponge)	not available	Hagadone *et al*., 1979
 9-isocyanopupukeanane: R^1= NC; R^2 = H 9-*epi*-9-isocyanopupukeanane: R^1= H; R^2 = NC	*P. bourguini* (nudibranch) *P. pustulosa* (nudibranch) *P.varicosa* (nudibranch) *Hymeniacidon* sp. (sponge)	not available	Fusetani *et al*., 1990 Fusetani *et al*., 1991 Burreson *et al*., 1975 Hagadone *et al*., 1979
 (-)-9-isothiocyanatopupukeanane: R = NCS (-)-9-isocyanopupukeanane: R = NC	*Axinyssa* sp. (sponge)	inactive for antimalarial activity	Simpson *et al*., 1997

Table 2. (cont.)

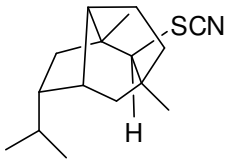
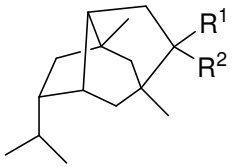
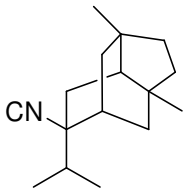
Structures	Sources	Biological activities	References
 2-thiocyanatopupukeanane	*Axinyssa aplysinoides* (sponge)	not available	He *et al*., 1992
 9-thiocyanatopupukeanane: R^1 = SCN; R^2 = H 9-*epi*-9-thiocyanatopupukeanane: R^1 = H; R^2 = SCN	*P. varicosa* (nudibranch)	not available	Yasman *et al.,* 2003
 9-isocyanoneopupukeanane	*Ciocalypta* sp.(sponge)	not available	Karuso *et al*., 1989

Table 2. (cont.)

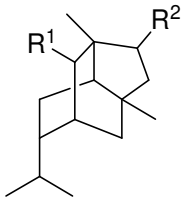
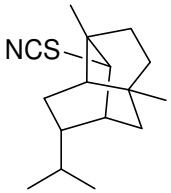
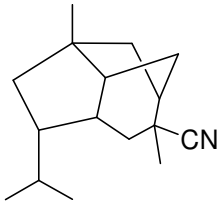
Structures	Sources	Biological activities	References
 2-thiocyanatoneopupukeanane: R^1 = SCN, R^2 = H 4-thiocyanatoneopupukeanane: R^1 = H, R^2 = SCN	*P. pustulosa* (nudibranch) *Phycopsis terpnis* (sponge)	2-thiocyanatoneopupukeanane: antifouling activity (IC_{50} = 4.6 μg/mL) and cytotoxic activity against KB (IC_{50} = 0.01 mg/mL) 4-thiocyanatoneopupukeanane: antifouling activity (IC_{50} = 2.3 μg/mL)	Okino *et al*., 1996 Pham *et al*., 1991
 (-)-2-thiocyanatoneopupukeanane	*Axinyssa* sp. (sponge)	inactive for antimalarial activity	Simpson *et al*., 1997

Table 2. (cont.)

Structures	Sources	Biological activities	References
 2-isocyanoallopupukeanane	*P. pustulosa* (nudibranch)	ichthyotoxic against *Oryziaslatipes* (LC_{50} = 10 μg/mL)	Fusetani *et al*., 1991

1.4 Secondary metabolites of nudibranchs

Nudibranchs or sea slugs, belonging to the order Nudibranchia, class Gastropoda, are a group of shell-less mollusks. As sources of secondary metabolites, nudibranchs are surprisingly one of the richest sources that yield a wide range of natural products. Very often, the secondary metabolites found in nudibranchs are actually not produced in the slugs themselves, but rather accumulated through their diets, which include sponges and algae. The compounds may be found either unaltered or transformed to certain extent in such a way that the nudibranch may tolerate such compounds or can store the sequestered metabolites more conveniently. In most cases, the collected metabolites are toxic components that were employed by the nudibranchs as their own chemical protections and weapons.

As mentioned, nudibranchs are the sources of toxic metabolites, and several metabolites from nudibranchs have potent biological activities with a good potential for the further development towards being used clinically. The milestone of the metabolites from nudibranchs was dolastatins from *Dolabella auricularia*. A derivative, dolastatin 10, which was in fact first isolated in 1993, (Pettit *et al*., 1993) was developed to link with specific monoclonal antibody, and was recently approved by US-FDA and European community under the name of brentuximab vendotin for the treatment of refractory Hodgkin's lymphoma. Another example of nudibranch metabolite currently in the clinical trial is jorunnamycins from *Jorunna funebris* (Charuphant *et al*., 2007). PM00104 was active against tumor cell lines and is now studied in phase I of clinical trial (Yep *et al*., 2012).

To date, there have been over hundreds of secondary metabolites ever isolated from the nudibranchs. To demonstrate the appreciation in such a wide variety of metabolites from nudibranchs, listed in Table 3 below are an excerpt of secondary metabolites isolated from nudibranchs reported over the past three years (2011-2014). The biological activities of the reported compounds, when applicable, are also included.

Table 3. Secondary metabolite of nudibranchs

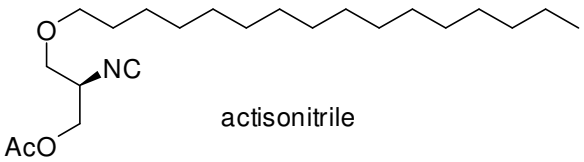
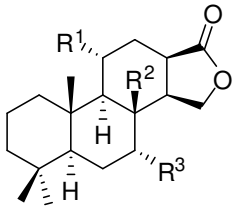
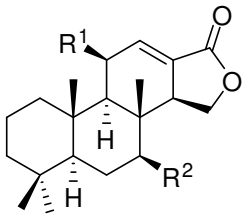
Structures	Sources	Biological activities	References
 actisonitrile	*Actinocyclus papillatus*	cytotoxic activity against nontumor H9c2 rat cardiac myoblast cells (IC_{50} = 23±6 μM)	Manzo *et al*., 2011
 11α-acetoxy-spongian-16-one: R^1 = OAc, R^2 = Me, R^3 = H spongian-16-one: R^1 = H, R^2 = Me, R^3 = H 7α-acetoxy-spongian-16-one: R^1 = H, R^2 = H, R^3 = OAc	*Chromodoris reticulata*	not available	Suciati *et al*., 2011
 dorisenone D: R^1 = R^2 = OAc 11β-hydroxyspongi-12-en-16-one: R^1 = OH, R^2 = H	*Chromodoris reticulata*	not available	Suciati *et al*., 2011

Table 3. (cont.)

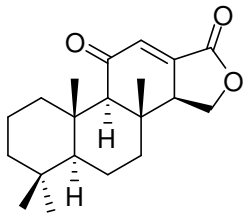
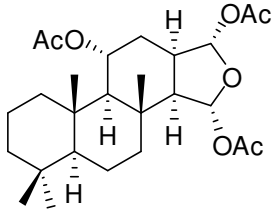
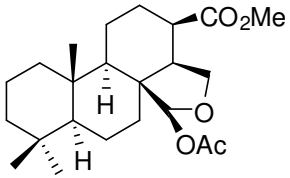
Structures	Sources	Biological activities	References
 spongi-12-en-11,16-dione	*Chromodoris reticulata*	not available	Suciati *et al*., 2011
 12-deacetyl-aplysillin	*Chromodoris reticulata*	not available	Suciati *et al*., 2011
 Methyl 15,17-epoxy-17α-acetoxy-*ent*-isocopalan16-oate	*Chromodoris reticulata*	not available	Suciati *et al*., 2011

Table 3. (cont.)

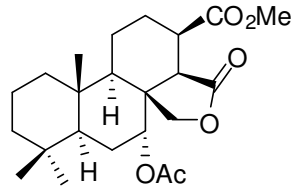
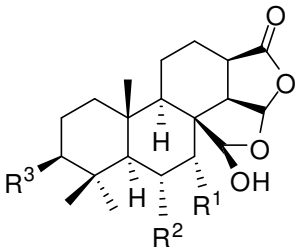
Structures	Sources	Biological activities	References
 7α-acetoxydendrillol-3	*Chromodoris reticulata*	not available	Suciati *et al*., 2011
 aplyroseols-2: $R^1 = R^3 = H$, $R^2 = OAc$ aplyroseols-3: $R^1 = OH$, $R^2 = OCOPr$, $R^3 = H$ aplyroseols-5: $R^1 = OCOPr$, $R^2 = OH$, $R^3 = H$ aplyroseol-19: $R^1 = OH$, $R^2 = OCOPr$, $R^3 = OAc$ dendrillol-1: $R^1 = R^2 = R^3 = H$ dendrillol-2: $R^1 = R^2 = OAc$, $R^3 = H$	*Chromodoris reticulata*	not available	Suciati *et al*., 2011

Table 3. (cont.)

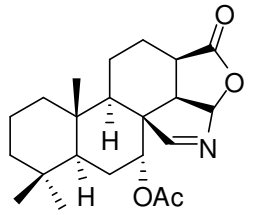
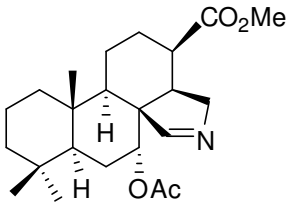
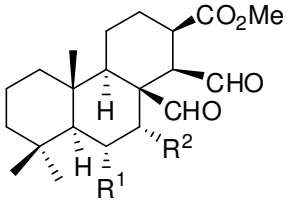
Structures	Sources	Biological activities	References
 chromoculatimine A	*Chromodoris reticulata*	not available	Suciati *et al*., 2011
 chromoculatimine B	*Chromodoris reticulata*	not available	Suciati *et al*., 2011
 methyl 6α-hydroxy-7α-butyryloxy-8β,14β–diformyl-podocarpane-13β-carboxylate: R^1 = OH, R^2 = OCOPr methyl-7α-acetoxy-8β,14β-diformylpodocarpane-13β–carboxylate: R^1 = H, R^2 = OAc	*Chromodoris reticulata*	not available	Suciati *et al*., 2011

Table 3. (cont.)

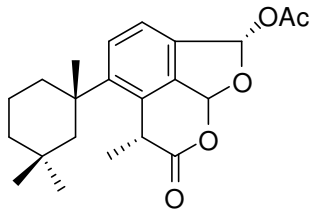
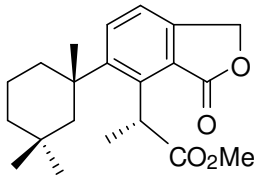
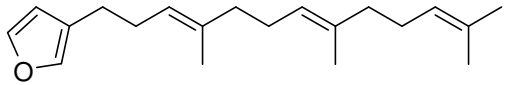
Structures	Sources	Biological activities	References
 aplysulfurin	*Chromodoris reticulata*	not available	Suciati *et al*., 2011
 membranolide	*Chromodoris reticulata*	not available	Suciati *et al*., 2011
 ambliofuran	*Chromodoris reticulata*	not available	Suciati *et al*., 2011

Table 3. (cont.)

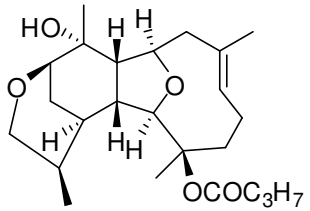
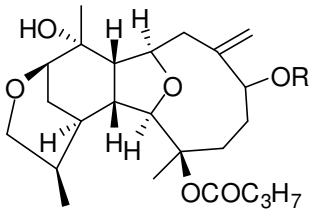
Structures	Sources	Biological activities	References
 tritoniopsin A	*Tritoniopsis elegans*	not available	Ciavatta *et al*., 2011
 tritoniopsin B: R = OH tritoniopsin C: R = H tritoniopsin D: R = Ac	*Tritoniopsis elegans*	tritoniopsin B: cytotoxicity activity against embryonic H9c2 rat cardiac myoblasts, 3T3-L1 murine fibroblasts, and Caco-2 human epithelial colorectal adenocarcinoma cells IC_{50} values ranging from 40 to 65 μM	Ciavatta *et al*., 2011

Table 3. (cont.)

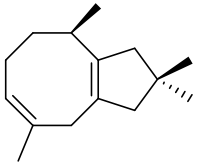
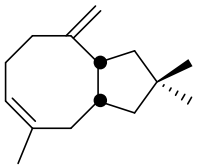
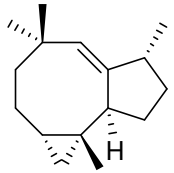
Structures	Sources	Biological activities	References
 asterisca-2(9), 6-diene	*Phyllodesmium magnum*	not available	Mao *et al*., 2011
 asterisca-3(15), 6-diene	*Phyllodesmium magnum*	not available	Mao *et al*., 2011
 1-africanene 2	*Phyllodesmium magnum*	not available	Mao *et al*., 2011

Table 3. (cont.)

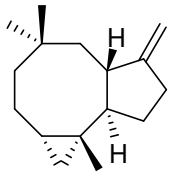
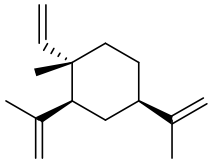
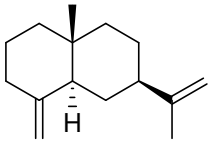
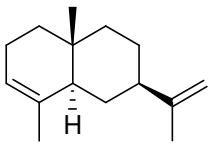
Structures	Sources	Biological activities	References
 9(15)-africanene	*Phyllodesmium magnum*	not available	Mao *et al*., 2011
 (-)β-elemene 4	*Phyllodesmium magnum*	not available	Mao *et al*., 2011
 (+)β-selinene	*Phyllodesmium magnum*	not available	Mao *et al*., 2011
 (-)-α-selinene	*Phyllodesmium magnum*	not available	Mao *et al*., 2011

Table 3. (cont.)

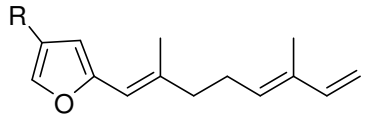
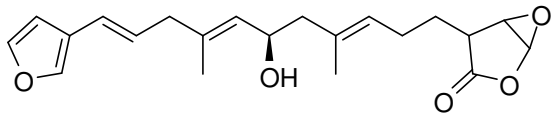
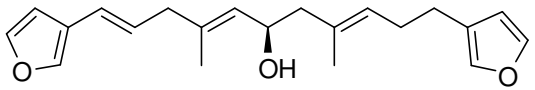
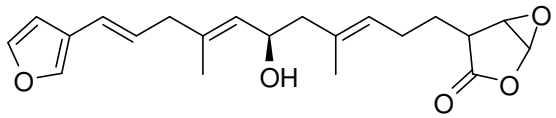
Structures	Sources	Biological activities	References
 2-[(2E,5E)-2,6-dimethylocta-2,5,7-trienyl]-4methylfuran: R = Me methyl 5-[(1E,5E)-2,6-dimethylocta-1,5,7-trienyl] furan-3-carboxylate: R = CO_2Me	*Phyllodesmium magnum*	not available	Mao *et al*., 2011
 epoxylactone	*Chromodoris albopunctata*	not available	Katavic *et al*., 2012
 (+)-tetradehydrofurospongin-1	*Chromodoris albopunctata*	not available	Katavic *et al*., 2012
 epoxylactone	*Chromodoris albopunctata*	not available	Katavic *et al*., 2012

Table 3. (cont.)

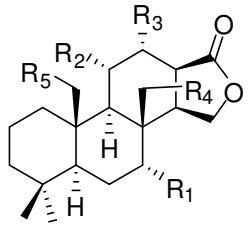
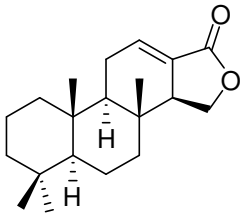
Structures	Sources	Biological activities	References
 R_1 R_2 R_3 R_4 R_5 spongian-16-one H H H H H spongian-16-one OAc H H H H spongian-16-one H H OAc H H spongian-16-one H H H H OAc spongian-16-one H H H H OCOEt spongian-16-one H H OCOEt H OCOEt	*Chromodoris albopunctata*	not available	Katavic *et al*., 2012
 (+)-isoagatholactone	*Chromodoris albopunctata*	not available	Katavic *et al*., 2012

Table 3. (cont.)

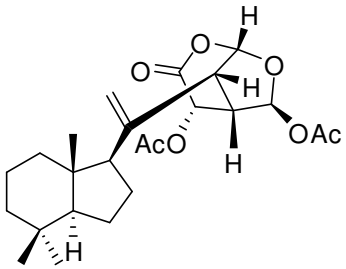
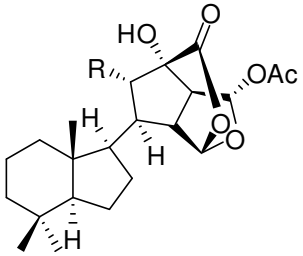
Structures	Sources	Biological activities	References
 norrlandin	*Chromodoris albopunctata*	not available	Katavic *et al*., 2012
 chromodorolides A: R = OAc chromodorolides A: R = H	*Chromodoris albopunctata*	not available	Katavic *et al*., 2012

Table 3. (cont.)

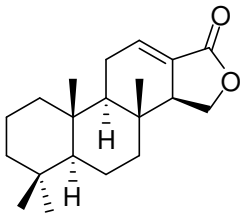
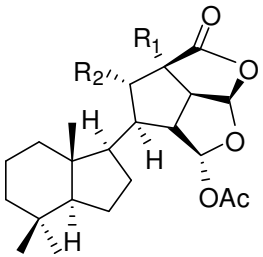
Structures	Sources	Biological activities	References
 (+)-isoagatholactone	*Chromodoris albopunctata*	not available	Katavic *et al*., 2012
 chromodorolide B: $R_1 = R_2 = OAc$ chromodorolide C: $R_1 = OH$, $R_2 = OAc$ chromodorolide E: $R_1 = R_2 = OH$	*Chromodoris albopunctata*	not available	Katavic *et al*., 2012

Table 3. (cont.)

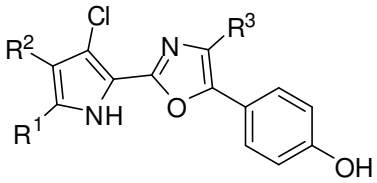
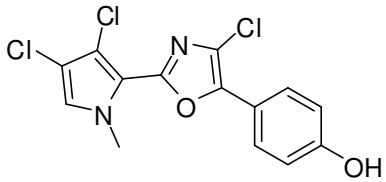
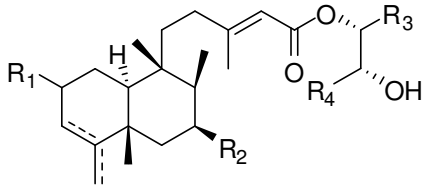
Structures	Sources	Biological activities	References
 phorbazole A: R^1 = H, R^2 = R^3 = Cl phorbazole B: R^1 = R^2 = Cl, R^3 = H phorbazole C: R^1 = R^2 = R^3 = H 2-chloro-phorbazole A: R^1 = R^2 = R^3 = Cl 9-chloro-phorbazole D: R^1 = R^2 = H, R^3 = Cl	*Aldisa andersoni*	2-chloro-phorbazole A, 9-chloro-phorbazole D and phorbazole A: feeding deterrence against the trophic generalist shrimp *Palaemon elegans* at a concentration of 1.0 mg/mL	Nuzzo *et al*., 2012
 *N*1-methyl-phorbazole A	*Aldisa andersoni*	not available	Nuzzo *et al*., 2012

Table 3. (cont.)

Structures	Sources	Biological activities	References
	*Austrodoris kerguelenensis*	Inhibits Jak2, STAT5, and Erk1/2 activation in human eryth roleukemia (HEL) cells,	Maschek *et al*., 2012

palmadorin	R_1	R_2	R_3	R_4	other
A	H	H	CH_2OH	H	$\Delta^{4,18}$
B	H	H	CH_2OAc	H	$\Delta^{4,18}$
C	H	OH	CH_2OH	H	$\Delta^{3,4}$
D	H	H	H	CH_2OH	$\Delta^{4,18}$
E	=O	OH	H	CH_2OH	$\Delta^{3,4}$
F	H	OH	H	CH_2OH	$\Delta^{3,4}$
G	=O	H	H	CH_2OH	$\Delta^{3,4}$
H	H	OH	CH_2OAc	H	$\Delta^{3,4}$
I	=O	=O	H	CH_2OH	$\Delta^{3,4}$
J	=O	OH	CH_2OH	H	$\Delta^{3,4}$
K	=O	=O	CH_2OH	H	$\Delta^{3,4}$

Palmadorin A (IC_{50} = 8.7±0.4 μM)

Palmadorin B (IC_{50} = 8.3±0.8 μM)

Palmadorin D (IC_{50} = 16.5±0.1 μM)

Table 3. (cont.)

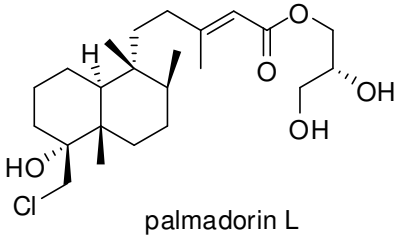
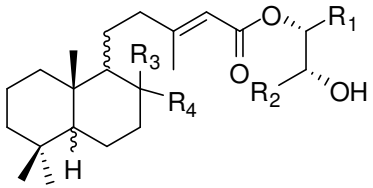
Structures	Sources	Biological activities	References
 palmadorin L	*Austrodoris kerguelenensis*	not available	Maschek *et al*., 2012
 palmadorin M: R_1 = CH_2OH, R_2 = H, R_3 = $=CH_2$, R_4 = –, other = 5β,9α palmadorin N: R_1 = H, R_2 = CH_2OH, R_3 = $=CH_2$, R_4 = –, other = 5β,9α palmadorin O: R_1 = CH_2OAc, R_2 = H, R_3 = $=CH_2$, R_4 = –, other = 5β,9α palmadorin P: R_1 = H, R_2 = CH_2OH, R_3 = βOH, R_4 = αCH_3, other = 5α,9β palmadorin Q: R_1 = H, R_2 = CH_2OH, R_3 = αOH, R_4 = βCH_3, other = 5α,9β	*Austrodoris kerguelenensis*	Inhibits Jak2, STAT5, and Erk1/2 activation in human eryth roleukemia (HEL) cells, Palmadorin M (IC_{50} = 4.9±0.4 μM) Palmadorin N (IC_{50} = 6.3±0.5 μM) Palmadorin O (IC_{50} = 13.4±0.4 μM)	Maschek *et al*., 2012

Table 3. (cont.)

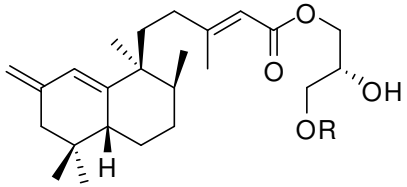
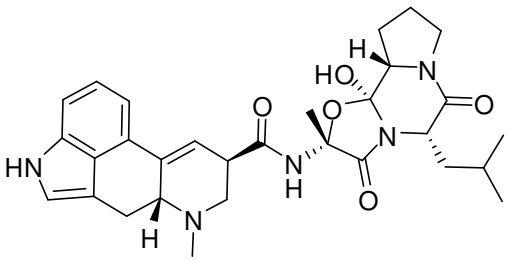
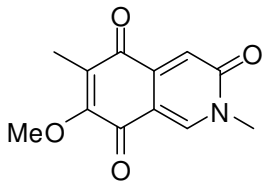
Structures	Sources	Biological activities	References
 palmadorin R: R = Ac palmadorin S: R = H	*Austrodoris kerguelenensis*	not available	Maschek *et al*., 2012
 ergosinine	*Pleurobranchus forskalii*	not available	Wakimoto *et al*., 2013
 mimosamycin	*Jorunna funebris*	not available	He *et al*., 2014

Table 3. (cont.)

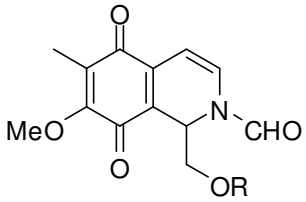
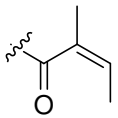
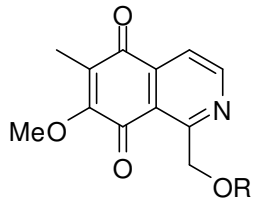
Structures	Sources	Biological activities	References
 *N*-formyl-1,2-dihydrorenierol acetate: R = Ac *N*-formyl-1,2-dihydrorenierone: R = 	*Jorunna funebris*	not available	He *et al*., 2014
 renierol: R = H renierol acetate: R = Ac	*Jorunna funebris*	not available	He *et al*., 2014

Table 3. (cont.)

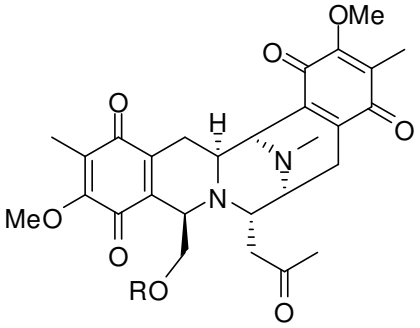
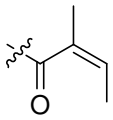
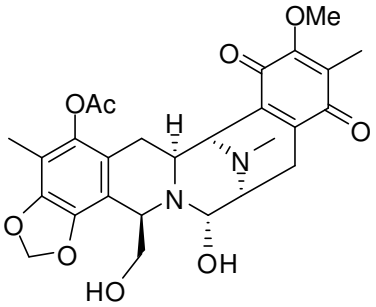
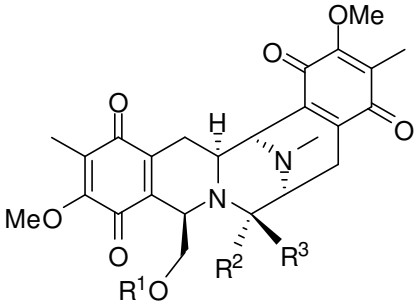
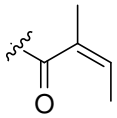
Structures	Sources	Biological activities	References
 fennebricins A: R = H renieramycin J: R = 	*Jorunna funebris*	not available	He *et al*., 2014
 fennebricins B	*Jorunna funebris*	not available	He *et al*., 2014

Table 3. (cont.)

Structures	Sources	Biological activities	References
 jorumycin: R^1 = OAc, R^2 = OH, R^3 = H renieramycin G: R^1 =  $R^2 = R^3 = O$	*Jorunna funebris*	not available	He *et al*., 2014

1.5 The genus *Phyllidia*

The nudibranchs of the genus *Phyllidia*, generally known as tubercle nudibranchs, belong to the family Phyllidiidae (order Nudibranchia). Easily recognizable by their ridged or pustulose dorsum with brightly colored spikes of the pustules, hence the name "tubercle", the slugs can be widely spotted throughout the tropical reefs, including most of coral reefs along Thai coastlines. Morphologically, the phyllidid nudibranch are characterized by the possession of a series of subnotal lamellae as gills instead of a circum-anal circlet on the upper surface of the mantle, as well as a foregut which lacks jaws and radula and is highly modified for auctorial feeding. Lack of these cuticularised buccal structures renders phyllidid nomenclature relatively more subjective than other families of the Anthobranchia. Currently six genera and about 50 biological species (80 nominal species) of Phyllidid nudibranchs are recognized (Brunckhorst, 1993; Willan *et al*., 1998; Chavanich *et al*., 2013).

1.5.1 *Phyllidia coelestis* Bergh

Live specimens of *P. coelestis* (Figure 11) range in length from 9 to 60 mm (average 33 mm. Description of *P. coelestis* Bergh was first given by Brunckhorst (1993), and was later modified by Willan *et al*. (1998), and Chavanich *et al*. (2013). The description is quoted below.

…*Phyllidia coelestis* Bergh is broad and oval in shape. The basic colours of *P. coelestis* Bergh are blue to grey-blue, black and yellow. The notum has three black longitudinal bands; two of these bands run laterally, each on the outside of two, mediolateral, blue-grey ridges which have tubercles capped in yellow. The ridges, which may be interrupted in some specimens, originate anteriorly behind each rhinophore and converge posteriorly in the region of the anus. Two tall, narrow, gold-capped rhinotubercles are present, one immediately behind each rhinophore. The rhinotubercles are separate from the two ridges. Two to four isolated, large, yellow capped tubercles arise from the median black band;

however, these are never joined as a midline ridge. There are always two isolated tubercles in the midline, one immediately anterior to, and the other immediately posterior to, the rhinophores. The rhinophores are yellow in colour and each clavus possesses 19-26 lamellae. Around the mantle margin is a broad blue-grey band with small tubercles, the larger ones of which may be yellow capped. Sometimes, meanderings of black encroach into this blue-grey margin. The foot is grey and has no distinctive markings on its sole... (Bruncкhorst, 1993).

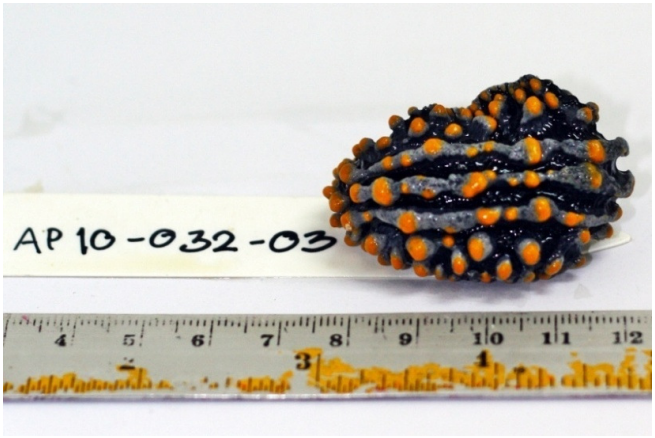


Figure 11. *Phyllidia coelestis* Bergh

1.6 Chemical constituents in the genus *Phyllidia*

The nudibranchs of the genus *Phyllidia* are well known as one of the sponge feeders, and have been reported to sequester specific sponge metabolites. One of the early observations of such dietary accumulation was in the slug *P. varicosa*, which was observed to secrete mucus that was lethal to crustaceans living in the same aquarium (Johannes, 1963). To date, there have been more than 30 compounds reported from the *Phyllidia* nudibranchs. Most of the compounds are non-alkaloidal nitrogenated sesquiterpenes, among which the predominant chemical skeletons are the rearranged sesquiterpenes of the pupukeanane family. The terpenoids were normally functionalized by the unusual isonitrile and related functionalities, including isocyanate, isothiocyanate and formamide. The pupukeananes and related sesquiterpenes are readily listed in Table 2. Summarized in Table 4 are additional sesquiterpenes of other classes that were reported from *Phyllidia* nudibranchs.

Table 4. The sesquiterpenes from *Phyllidia* spp.

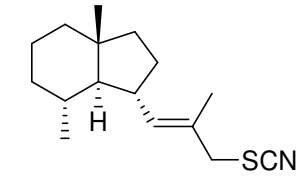
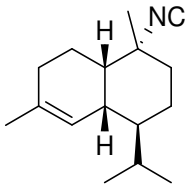
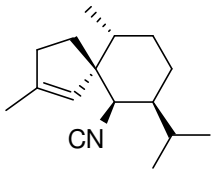
Structures	Nudibranchs	Biological activities	References
 cavemothiocyanate	*P. ocelkzta*	not available	Fusetani *et al*., 1992
 10α-isocyan-4-amorphane	*P. ocelkzta*	not available	Fusetani *et al*., 1992
 axisonitrile-3	*P. ocelkzta*	not available	Fusetani *et al*., 1992

Table 4. (cont.)

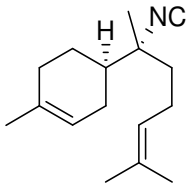
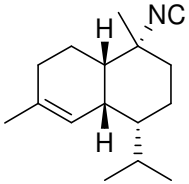
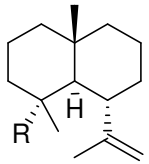
Structures	Nudibranchs	Biological activities	References
 7-isocyano-7,8-dihydro-α-bisabolene	*P. ocelkzta*	not available	Fusetani *et al*., 1992
 10-isocyano-4-amorphane	*P. ocelata*	antifouling activity (IC_{50} = 0.70 μg/mL)	Okino *et al*., 1996
 4α-isocyanogorgon-11-ene: R = NC 4α-formamidogorgon-11-ene: R = NHCHO 4α-isothiocyanatogorgon-11-ene: R = NCS	*P. pustulosa*	not available	Kassuhlke *et al*., 1991

Table 4. (cont.)

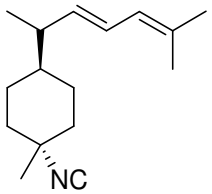
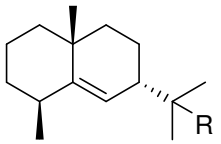
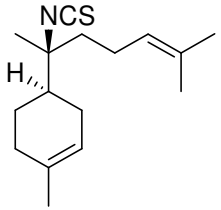
Structures	Nudibranchs	Biological activities	References
 3-isocyanobisabolane-8,10-diene	*P. pustulosa*	not available	Kassuhlke *et al*., 1991
 11-isocyano-7β-*H*-eudesm-5-ene: R = NC 11-isothiocyanato-7β-*H*-eudesm-5-ene: R = NCS	*P. pustulosa*	not available	Kassuhlke *et al*., 1991
 6*R*,7*S*-7-isothiocyanato-7,8-dihydro-α-bisabolene	*P. pustulosa*	not available	Kassuhlke *et al*., 1991

Table 4. (cont.)

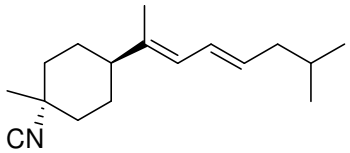
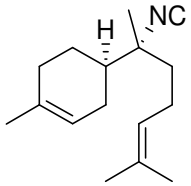
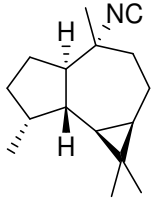
Structures	Nudibranchs	Biological activities	References
 isocyanotheonellin	*P. pustulosa*	not available	Fusetani *et al*., 1991
 7-isocyano-7,8-dihydro-α-bisabolene	*P. pustulosa*	not available	Fusetani *et al*., 1991
 axisonitrile-2	*P. pustulosa*	not available	Fusetani *et al*., 1991

Table 4. (cont.)

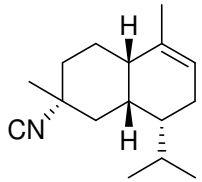
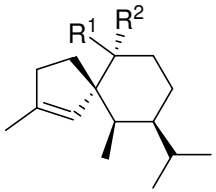
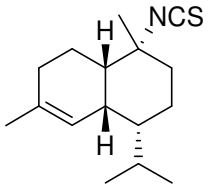
Structures	Nudibranchs	Biological activities	References
 4α-isocyano-9-amorphane	*P. pustulosa*	not available	Fusetani *et al*., 1991
 10-*epi*-axisonitrile-3: $R^1 = CH_3$, $R^2 = H$ axisonitrile-3: $R^1 = H$, $R^2 = CH_3$	*P. pustulosa*	antifouling activity, 10-*epi*-axisonitrile-3 (IC_{50} = 10 μg/mL), axisonitrile-3 (IC_{50} = 3.2 μg/mL)	Okino *et al*., 1996
 (-)-10-isothiocyanato-4-amorphane		(-)-10-isothiocyanato-4-amorphane (IC_{50} = 7.2 μg/mL)	

Table 4. (cont.)

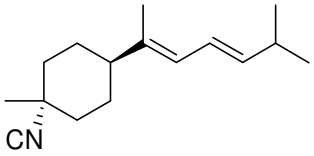
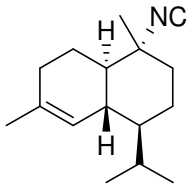
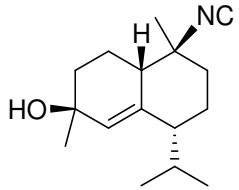
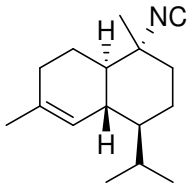
Structures	Nudibranchs	Biological activities	References
 3-isocyanotheonellin	*P. pustulosa*	antifouling activity (IC_{50} = 0.13 μg/mL)	Okino *et al*., 1996
 10-isocyano-4-cadinene	*P. pustulosa*	antifouling activity (IC_{50} = 0.14 μg/mL)	Okino *et al*., 1996
		inactive for antifouling activity	
 10-isocyano-5-cadinen-4-ol	*P. pustulosa*		Hirota *et al*., 1998

Table 4. (cont.)

Structures	Nudibranchs	Biological activities	References
 10-isocyano-4-cadinene	*P. varicosa*	antifouling activity (IC_{50} = 0.14 µg/mL)	Okino *et al*., 1996

1.7 Rationales and objectives

The nudibranch *Phyllidia coelestis* Bergh is one of tubercle nudibranchs roaming coral reefs along Thai coastlines. As a well-known sponge feeders that can sequester sponge metabolites and employ such compounds as the chemical defense, it is of interest to explore the chemical constituents in the nudibranch. Finding of any sponge-related metabolites in the collected specimens may not only lead to a discovery of new bioactive compounds, but also open up an opportunity to explore the chemical relation between predating slugs and sponge prays. The aims of this project are;

1) To purify and elucidate the compounds of the chemical constituents of the nudibranch *Phyllidia coelestis* Bergh.

2) To determine the biological activities of the isolated compounds.

CHAPTER 2

EXPERIMENTAL

2.1 General experimental procedures

Unless stated otherwise, all chemicals are used as purchased. All solvents for general purposes and for chromatographic separation were of commercial grade and were re-distilled prior to use. TLC was performed on silica gel 60 F_{254} plates (0.20 mm thickness; Merck®). SiO_2 for chromatographic separations was SiO_2 60 (particle size 0.04-0.06 mm; Merck®). Size exclusion chromatography was performed over Sephadex LH-20 (bead size 25-100 μm; Merck®). Visualization was done with observation under UV light (254 nm), and with anisaldehyde/H_2SO_4 and phosphomolybdic acid spraying reagents. Melting point was recorded on a Fisher-Johns melting point apparatus. Optical rotations were measured on a Perkin-Elmer 34J polarimeter (Faculty of Pharmaceutical Sciences, Chulalongkorn University). UV spectra were recorded on a Genersys 6 spectrophotometer. ECD spectra were measured on a Jasco J-715 spectropolarimeter. IR spectra were recorded on a Jasco 810 IR spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on an FT-NMR, Varian® Unity Inova 500 spectrometer. Chemical shifts are recorded relative to the solvent signals of $CDCl_3$ (7.24 ppm of residual $CHCl_3$ for ^{1}H NMR, and 77.0 ppm for ^{13}C NMR). The EI-MS was performed using an MAT 95 XL mass spectrometer. HPLC was performed on a Thermo Scientific SCM1000 solvent delivery system, connected to a SPECTRA system 1500 diode UV tunable detector and a Rheodyne V77251 injector port.

For the chemical reactions acetonitrile and diethylether were anhydrous and high-purity research graded and were used without additional purification. All chemical reactions were conducted under dried nitrogen atmosphere in oven-dried, flame-dried vessels, using either oven-dried or seal-tight disposable glassware.

2.2 Animal material

Eight *Phyllidia coelestis* Bergh nudibranchs were collected using SCUBA at the depth of 15-20 metres, from Koh-Ha Islets, Krabi Province (7° 25′ 46.7″ N, 98° 53′ 41.5″ E), Thailand, in February, 2010. The slugs were preserved in an ice-chest upon surfacing, and at -20 °C once arrived at the lab until extraction. One specimen (AP10-032-03) was preserved and deposited at Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand. The nudibranch was identified as *Phyllidia coelestis* Bergh (family Phyllidiidae, order Nudibranchia) by Assoc. Prof. Dr. Suchana Chawanich of Department of Marine Science, Faculty of Science, Chulalongkorn University, and Bangkok, Thailand.

2.3 Extraction and isolation

Seven specimens of *P. coelestis* Bergh were homogenized and macerated exhaustively in MeOH/EtOAc (1:1) (3 × 20 mL). The concentrated extract (0.7 g), after partitioning in EtOAc to eliminate trace water, was further partitioned with a series of solvents to yield *n*-hexane-, $CHCl_3$-, and *n*-BuOH-extracts (457, 44, and 24 mg), respectively.

The hexane-extract was fractionated over a SiO_2 column (CH_2Cl_2/hexane 9:1) then a Sephadex LH-20 (CH_2Cl_2/MeOH 1:1) column. The major fraction was pooled and chromatographed over a SiO_2 HPLC column (VertiSep, 10 × 250 mm, 10 mm; 3% *i*-PrOH in hexane) to yield **1** and **2** (2.1 and 1.6 mg, respectively). The fraction that contained **1** was allowed to crystallize in the same chromatographic solvent to yield crystals of **1**.

1-Formamido-10(1⟶2)-abeopupukeanane (1): colorless, orthorhombic crystals; mp 152 °C; $[\alpha]_D$ +17.2 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (logε) 293 (2.18) nm; ECD (*c* 0.10, MeOH) λ ($\Delta\varepsilon$) 295 (-4.5), 228 (-4.3) nm; (*c* 0.10, $CHCl_3$) λ ($\Delta\varepsilon$) 230 (-8), 222 (6.5), 215 (-2.5) nm; IR (neat) ν 3290, 2930, 1655 cm^{-1}; ^1H and ^{13}C NMR see Table 4; HREIMS *m/z* 249.2087 $[M]^+$ (calcd for $C_{16}H_{27}NO$, 249.20926).

2-Formamidopupukeanane (2): colorless solid; $[\alpha]_D$ -36 (*c* 0.08, MeOH); UV (MeOH) λ_{max} (logε) 290 (2.08) nm; ECD (*c* 0.08, MeOH) λ ($\Delta\varepsilon$) 295 (-3.5), 220 (-11) nm; (*c* 0.08, $CHCl_3$) λ ($\Delta\varepsilon$) 230 (-10.5), 220 (7.5) nm; IR (neat) ν 3300, 2950, 1662 cm^{-1}; ^1H and ^{13}C NMR see Table 5; HREIMS *m/z* 249.2087 $[M]^+$ (calcd for $C_{16}H_{27}NO$, 249.20926).

2.4 X-ray crystallography

The crystallographic experiments were kindly supported by Assoc. Prof. Palangpon Kongsaeree, Department of Chemistry and Center of Excellence in Protein Structure and Function, Faculty of Science, Mahidol University, Bangkok, Thailand. X-ray diffraction data were obtained on a Bruker-Nonius kappa CCD diffractometer with graphite monochromated MoKα radiation (λ = 0.71073 Å) at 150(2) K. The structure was solved with direct methods by SIR97 and refined with full-matrix least-squares calculations on F^2 using SHELXL-97. Crystallographic data have been deposited at the Cambridge Crystallographic Data Centre under the reference number CCDC 915518.

1-Formamido-10(1⟶2)-abeopupukeanane (1): $C_{16}H_{27}NO$, orthorhombic, dimensions 0.30 × 0.15 × 0.10 mm^3, D = 1.139 g/cm^3, space group $P2_12_12_1$, Z =4, a = 7.0316(2) Å, b = 9.5125(4) Å, c = 21.7347(8) Å, V = 1453.79(9) $Å^3$, refractions collected/unique: 8819/3794, number of observations [>2σ(I)] 3426,final R indices [I >2σ(I)] R_1 = 0.0460, wR_2 = 0.1124.

2.5 Density-functional theory calculations

The geometrical parameters of molecules are determined in the solid phase calculation. The input structures of each molecule are calculated by GaussView 3.0 to obtain optimized low-energy conformers. For the spectral simulation, the Gaussian 03 is used to predict sufficient properties of 1-formamido-10(1⟶2)-abeopupukeanane (**1**). All low energy minima with internal energies were optimized with Hartree-Fock method at B3LYP/6-31G(d) level. Time-dependent density-functional theory (TDDFT) calculations were conducted using a function PDB1PDB and basis set at B3LYP/6-31G(d) level.

2.6 Synthesis of tricyclic analogues

Amidation of α-pinene was carried out according to the protocol described by Subba *et al* (2010). To a mixture of (-)-α-pinene (200 mg) and $HBF_4.OEt_2$ (285 mg) was added either MeCN (72 mg) or *n*-PrCN (121 mg). The mixture of each reaction was separately stirred at an ambient condition. Upon the complete consumption (2 h), $NaHCO_3$ was added. The reaction mixture was extracted (×3) with EtOAc and was chromatographed over SiO_2 columns (EtOAc/hexane 1:4 for both **3** and **4**).

8-Acetamido-2,4,4,8-tetramethyl-3-azabicyclo[3.3.1]-non-2-ene (3): (137 mg; 48%), yellow solid; ECD (*c* 0.10, MeOH) λ ($\Delta\varepsilon$) 245 (8.9), 215 (-2.2) nm; (*c* 0.10, $CHCl_3$) λ ($\Delta\varepsilon$) 248 (-8.8), 227 (9.9), 220 (-8.9) nm; ^{1}H-NMR (500 MHz, $CDCl_3$): 5.54 (br s, NH), 3.12 (br d,1.5: H-1), 2.01 (s, H_3-12), 1.92 (s, H_3-14), 1.74 (m, 14.5: H_{eq}-4), 1.70 (ddd,13.5, 3.0, 1.5: H_{ax}-7), 1.65 (ddd,13.5, 6.0, 3.0: H_{eq}-7), 1.56 (m, H-5), 1.52 (tt, 14.5, 4.5: H_{ax}-4), 1.44 (m, 14.5: H_{eq}-3), 1.38 (s, H_3-10), 1.33 (td, 14.5, 5.0: H_{ax}-3), 1.19 (s, H_3-8), 1.07 (s, H_3-9); ^{13}C-NMR (125 MHz, $CDCl_3$): 169.7 (C-13), 166.9 (C-11), 57.9 (C-6), 55.6 (C-2), 40.2 (C-1), 33.8 (C-5), 33.1 (C-3), 31.6 (C-9), 29.7 (C-12), 27.2 (C-10), 26.3 (C-8), 24.5 (C-4), 24.46 (C-14), 24.0 (C-7); HREIMS: *m/z* 236.1880 $[M]^+$ (calcd for $C_{14}H_{24}N_2O$, 236.1889).

8-Propylamido-2-propyl-4,4,8-trimethyl-3-azabicyclo[3.3.1]-non-2-ene (4): (183 mg; 82%), yellow solid; ECD (*c* 0.10, MeOH) λ ($\Delta\varepsilon$) 245 (8.7), 215 (-6.2) nm; (*c* 0.10, $CHCl_3$) λ ($\Delta\varepsilon$) 230 (9.3), 215 (-3.9) nm; ^{1}H-NMR (500 MHz, $CDCl_3$): δ 5.72 (s, NH), 3.59 (s, H-1), 2.40 (td, 8.0, 1.5: H_2-12), 2.14 (td,7.5, 3.0: H_2-16), 1.83 (dt,13.0, 2.5: H_{ax}-7), 1.78 (m, H-4), 1.68 (m, H_{eq}-7), 1.67 (m, H-5), 1.66 (m, H-4), 1.61 (m, H_2-17), 1.56 (m, H-3), 1.50 (m, H_2-13), 1.39 (s, H_3-10), 1.34 (s, H_3-8), 1.32 (m, H-3), 1.21 (s, H_3-9), 0.91 (t, 7.5: H_3-14), 0.91 (t, 7.5: H_3-18); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 178.3 (C-11), 173.4 (C-15), 59.3 (C-6), 55.7 (C-2), 43.2 (C-12), 39.0 (C-16), 38.7 (C-1), 33.8 (C-5), 32.5 (C-3), 30.9 (C-9), 26.8 (C-10), 25.0 (C-8), 24.0 (C-4), 23.5 (C-7), 21.8 (C-13), 19.0 (C-17), 13.6 (C-14), 13.6 (C-18); HREIMS: *m/z* 292.2509 $[M]^+$ (calcd for $C_{18}H_{32}N_2O$, 292.2515).

2,6,6,8-Tetramethyl-7-azabicyclo[3.3.1]-non-2-ene (5): (36 mg; 12%), ^{1}H-NMR (500 MHz, $CDCl_3$): δ5.35 (m: H-3), 2.56 (m: H-1), 2.22 (m, 17.5: Ha-4), 2.14 (m, 17.5: Hb-4), 1.86 (m: H-5), 1.86 (m: Ha-9) 1.78 (dd, 4.0, 2.0: H_3-10), 1.56 (ddd, 13.5, 3.5, 2.5: Hb-9), 1.21 (s: H_3-11), 1.15 (s: H_3-12), 2.08 (s: H_3-13); ^{13}C-NMR (125 MHz, $CDCl_3$): δ170.5 (C-8), 133.8 (C-2), 122.9 (C-3), 58.9 (C-6), 40.3 (C-1), 33.2 (C-5), 31.2 (C-11), 28.6 (C-4), 27.7 (C-13), 27.4 (C-12), 25.0 (C-9), 23.7 (C-10); HREIMS: *m/z* 177.1517 $[M]^+$ (calcd for $C_{12}H_{19}N$, 177.2859).

2,6,6-Trimethyl-8-propyl-3-azabicyclo[3.3.1]-non-2-ene (6): (21 mg; 10%), ^{1}H-NMR (500 MHz, $CDCl_3$): δ5.64 (m: H-3), 3.15 (br s: H-1), 2.88 (m: Hb-3), 2.60 (m: Ha-13), 2.40 (m: Hb-4), 2.32 (m: Ha-4), 2.13 (m: H-5), 2.04 (m: Hb-14), 2.02 (m: Hb-9), 1.89 (m: Ha-14), 1.82 (dd, 4.0, 2.5: H_3-10), 1.79 (m: Ha-9), 1.50 (s: H_3-11), 1.44 (s: H_3-12), 1.01 t (7.5); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 190.5 (C-8), 130.7 (C-2), 126.7 (C-3), 62.0 (C-6), 39.7 (C-13), 38.7 (C-1), 33.3 (C-5), 29.4 (C-11), 28.2 (C-4), 24.9 (C-12), 23.4 (C-9), 23.2 (C-10), 20.1 (C-14), 13.4 (C-15); HREIMS: *m/z* 205.18305 $[M]^+$ (calcd for $C_{14}H_{23}N$, 205.18315).

2.7 Cytotoxic activity determination

The SRB assay (Skehan *et al*., 1990) for the cytotoxicity determination was kindly supported by Assist. Prof. Dr. Supreeya Yuenyongsawad, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University. The four cancer cell lines targeted here were MCF-7 (breast cancer), HeLa (cervical cancer), KB (oral cancer) and HT-29 (colon cancer) cells, and human gingival fibroblasts were used to represent the normal cells.

Briefly, monolayered culture of each all line in a 96-well microtiter plate (2×10^3 cells/well) was treated with a serial dilution (at least five concentrations) of each sample in a suitable culture medium. All the plates were incubated according to the reported condition, for seven days, at midway of which time the medium was refreshed once (exposure time 72 h). Ice-cold 40% TCA was added. The plates were washed with water (×5). The TCA-fixed cells were stained for 30 min with 0.4% SRB in 1% acetic acid. The plates were washed with 1% acetic acid (×5) and allowed to dry overnight. Once dried, bound dye was dissolved with 10 mM Tris for 20

min on a gyratory shaker. Survival percentage was measured via the intensity of the resulted purplish-pink color at 492 nm (Power Wave X plate reader). The IC_{50} values were calculated from the dose-response curves. Camptothecin was used as a standard drug (IC_{50} 0.13 ± 0.003, (3.3 ± 1.1) × 10^{-3}, 0.02 ± 0.003, and (0.7 ± 0.1) × 10^{-3} μM against HeLa, MCF-7, KB, and HT-29 cancer cells, respectively).

CHAPTER 3

RESULTS AND DISCUSSION

The goal of this investigation is to establish a chemical library for the marine-derived sesquiterpenes, namely the pupukeanane derivatives, which are a rare class of rearranged, bridged sesquiterpenes. The biological activity assessed here is the cytotoxicity. The discussion in this chapter is to be presented in three main parts; the isolation and structure determination of the sesquiterpenes from the nudibranch *Phyllidia coelestis*, the attempts to determine the absolute configurations of the isolated compounds, and the biological activities.

3.1 Isolation and structure elucidation

3.1.1 The isolation

The hexane-extract from *Phyllidia coelestis* Bergh was fractionated chromatographically to yield two compounds, **1** and **2** (2.1 mg, and 1.6 mg, respectively). Compound **1** was allowed to crystallize, and orthorhombic crystals were obtained.

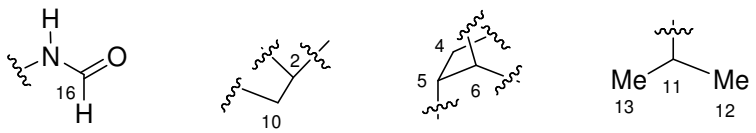
3.1.1.1 1-Formamido-10(1⟶2)-abeopupukeanane (1)

Compound **1** was obtained as orthorhombic crystals from 3% *i*-PrOH in hexane. The molecular formula of $C_{16}H_{27}NO$ was established by the high resolution EI mass spectrum, which also showed a peak of $[M]^+$ at *m/z* 249.2087 (calcd for $C_{16}H_{27}NO$, 249.2093). The proposed molecular formula requires the unsaturation degree of 4, one of which is a carbonyl; hence three rings are needed. The infrared absorptions at ν 3290 and 1655 cm^{-1} belong to a secondary amine and an amide carbonyl stretching. Combining with the formyl carbon at δ 163.2 in the ^{13}C NMR spectrum, these indicate the presence of a formamide functionality. The UV spectrum shows the maxima at λ 293 nm. This is a bathochromic shift caused by the solvent effect from H-bond between the formamide group and MeOH.

Two sets of NMR resonances in an approximately 1:1 ratio were found both in the ^{1}H and ^{13}C NMR spectra. This is coherent with the presence of the formamide functionality. The slow rotation about the amide bond results in the *cisoid* and *transoid* conformations. The formamide functional group was observed at δ 8.20 (d, *J* = 12.6 Hz, *transoid*) and 8.02 (d, *J* = 2.1 Hz, *cisoid*) for H-16 and at δ 5.58 (br d, *J* = 12.6 Hz, *transoid*) and 5.15 (br s, *cisoid*) for 1-N*H*. The corresponding carbonyls resonate at δ 163.2 (C-16, *transoid*) and 160.8 (C-16, *cisoid*). Similar NMR phenomenon of the formamide rotamers have been reported elsewhere (for examples, see Chanthathamrongsiri *et al*., 2012; Dalisay and Molinski, 2010; Hirota *et al*., 1996; Patil *et al*., 1997). For brevity, the discussion in this dissertation refers to the conformer relating to the signals resonate at δ 8.02 (d, *J* = 2.1 Hz, H-16) and 5.15 (br s, 1-N*H*) of *cisoid* conformation. The according chemical shifts of the *transoid* conformer are shown in Table 5.

The ^{1}H-NMR spectrum (Figure 12) of **1** exhibited a doublet of two methyl groups (δ 0.80, d, *J* = 6.5 Hz, 6H, H_3-12 and H_3-13), two aliphatic methyl singlets (δ 1.24, H_3-14; 1.30, H_3-15), one formamide proton (δ 8.02, d, *J* = 2.1 Hz, H-16) and one N*H* proton (δ 5.15, d, *J* = 2.1 Hz), along with the remaining resonances of the aliphatic methylene and methine protons clustering densely in the high-field region (δ_H 0.75-2.05). The ^{13}C-NMR spectrum (Figure 13) revealed 16 carbons including one formyl, two quaternary carbons, five methines, four methylenes, and four methyls.

Elucidating the overlapping signals of all the aliphatic methylenes and methines relied heavily on the analysis of the COSY spectrum. Four fragments that can be clearly identified are as followed: fragment **A**, between δ_H 8.02 (d, *J* = 2.1 Hz, H-16) and 5.15 (br s, 1-N*H*); fragment **B**, between δ_H 2.02 (br d, *J* = 8.0 Hz, H-2) and 1.47 (m, H_2-10); fragment **C**, from δ_H 1.44 (m, H-4β) and 0.91 (m, H-4α) through 1.26 (m, H-5) to 1.94 (dd, *J* = 5.5, 2.5 Hz, H-6), and fragment **D**, among δ_H 1.42 (m, H-11) and δ_H 0.80 (d, *J* = 6.5 Hz, 6H, H_3-12 and H_3-13).



A **B** **C** **D**

Table 5. ^{1}H and ^{13}C NMR spectral data of **1** (500 MHz for ^{1}H, 125 MHz for ^{13}C; $CDCl_3$)

Position	δ_H (J in Hz)		δ_C	
	cisoid	*transoid*	*cisoid*	*transoid*
1	-	-	57.8 (C)	56.3
2	2.02 br d (8.0)	1.51 m	49.8 (CH)	53.4
3	-	-	45.6 (C)	45.7
4α	0.91 m	0.91 m	45.3 (CH_2)	45.3
4β	1.44 m	1.44 m	-	-
5	1.26 m	1.26 m	50.2 (CH)	50.1
6	1.94 dd (5.5, 2.5)	1.98 dd (5.5, 2.5)	41.3 (CH)	41.5
7	1.45 m	1.40 m	49.4 (CH)	49.1
8α	1.73 m	1.73 m	20.6 (CH_2)	20.2
8β	1.58 m	1.58 m	-	-
9α	1.62 m	1.62 m	30.5 (CH_2)	31.0
9β	1.72 m	1.72 m	-	-
10α	1.47 m	1.52 m	24.5 (CH_2)	24.9
10β	1.22 m	1.24 m	-	-
11	1.42 m	1.42 m	28.8 (CH)	28.8
12	0.80 d (6.5)	0.80 d (6.5)	21.6 (CH_3)	21.4
13	0.80 d (6.5)	0.80 d (6.5)	21.6 (CH_3)	21.5
14	1.24 s	1.19 s	19.9 (CH_3)	21.2
15	1.30 s	1.21 s	25.6 (CH_3)	30.3
16	8.02 d (2.1)	8.20 d (12.6)	163.2 (CH)	160.8
1-N*H*	5.15 br s	5.58 d (12.6)	-	-

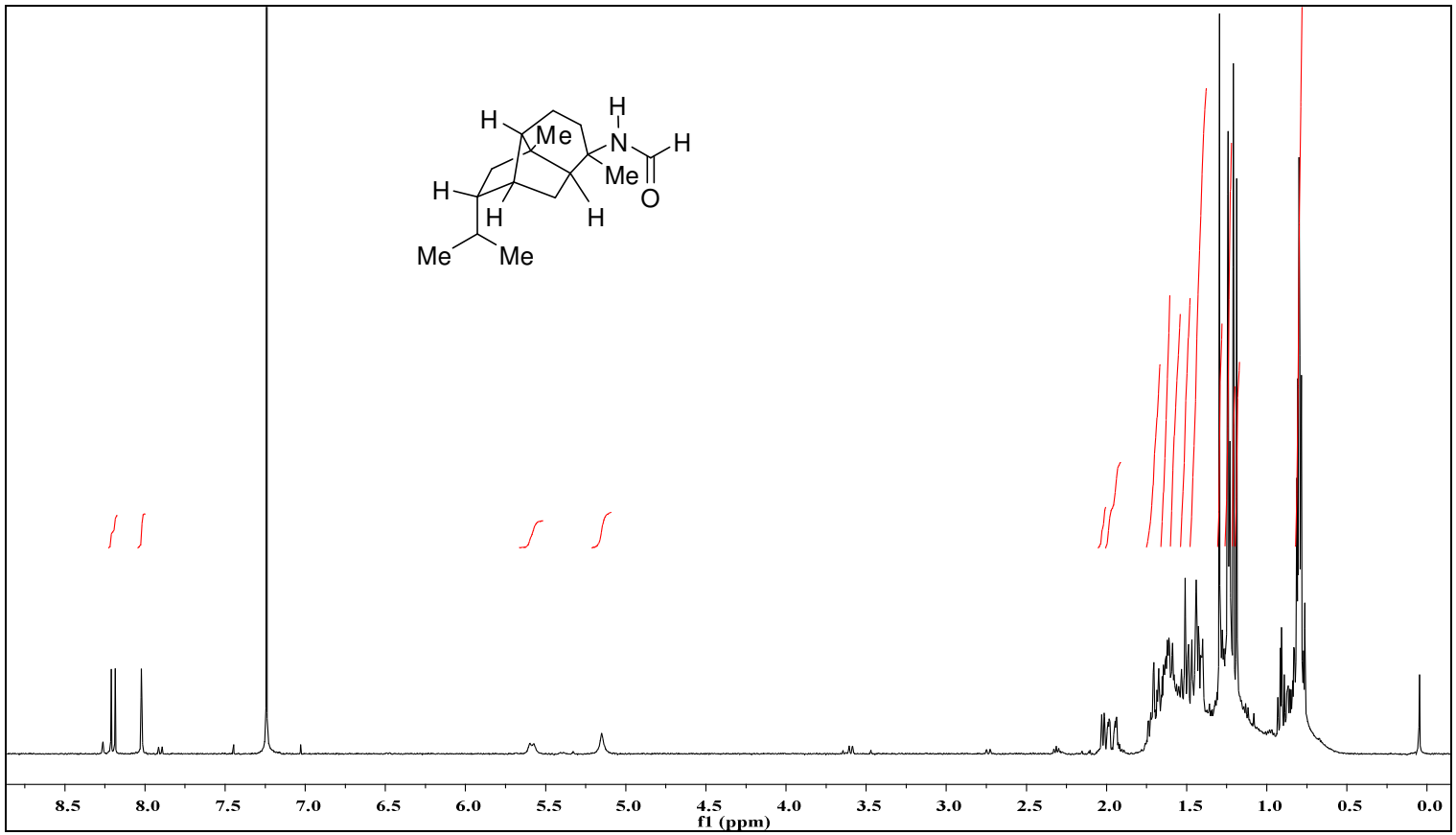


Figure 12. ^{1}H-NMR spectrum of **1** (500 MHz, $CDCl_3$)

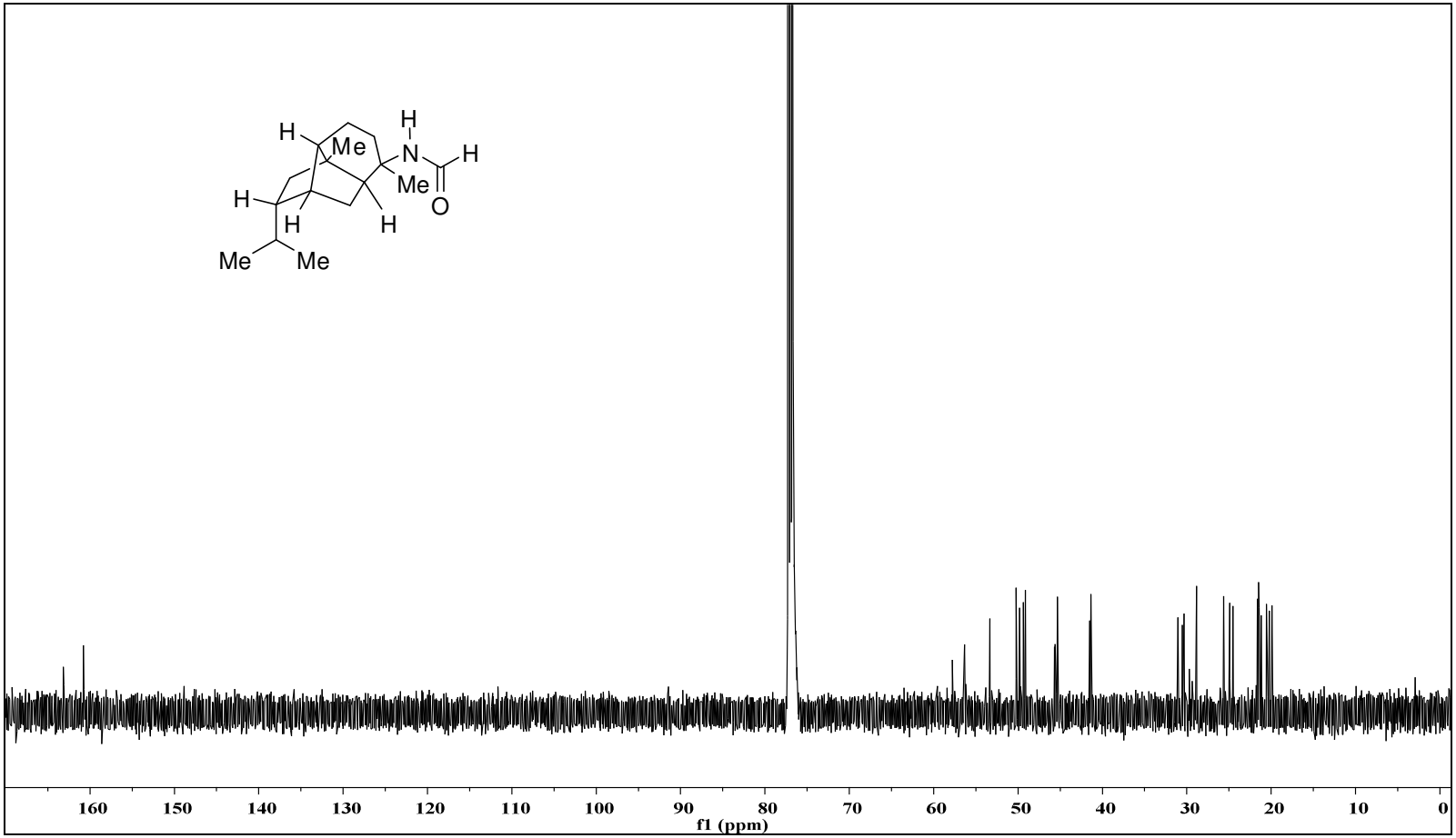


Figure 13. ^{13}C-NMR spectrum of **1** (125 MHz, $CDCl_3$)

Having three ring systems as indicated by the unsaturation degree, the three aliphatic quaternary carbons are expected to constitute either the bridgehead or the ring junction. HMBC correlations were analyzed and allowed the connection of the four fragments described earlier over the three quaternary carbons (Figure 14). The crucial correlations include those from δ_H 1.94 (dd, *J* = 5.5, 2.5 Hz, H-6), 1.44 (m, H-4β), 1.30 (3H, s, H_3-15), 1.24 (3H, s, H_3-14), and 0.91 (m, H-4α) to δ_C 49.8 (C-2); from δ_H 2.02 (br d, *J* = 8.0 Hz, H-2) 1.44 (m, H-4β), 1.26 (m, H-5), 1.24 (3H, s, H_3-14), and 0.91 (m, H-4α) to δ_C 45.6 (C-3); from δ_H 1.47 (m, H-10), 1.42 (m, H-11), and 1.26 (m, H-5) to δ_C 41.3 (C-6); and from δ_H 1.62 (2H, m, H_2-9), 1.47 (m, H-10), and 1.26 (m, H-5) to δ_C 49.4 (C-7). A core structure of tricyclo[4.4.0.$0^{2,8}$]decane is therefore constructed. The isopropyl and *N*-formyl moieties were placed on C-5 and C-1 according to the HMBC correlation from H-11 and N*H*, respectively. The structure of **1** is therefore proposed here as 1-formamido-10(1⟶2)-abeopupukeanane, a new rearranged sesquiterpene of the pupukeanane family.

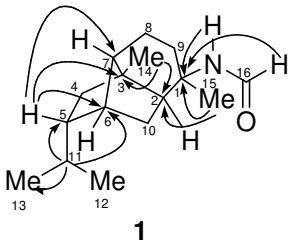


Figure 14. Selected HMBC correlations of **1**

The proposed structure of **1** was confirmed by X-ray crystallographic analysis (Figure 15). The crystallographic data also revealed the relative configuration of **1** as 1*S**,2*S**,3*S**,5*S**,6*R**,7*R**. The conformation of the formamide moiety was determined based on the analysis of the coupling constants between the amide and formyl protons (2.1 Hz for δ 8.02 vs. 12.6 Hz for δ 8.20). The signals related to the smaller *cisoid* coupling are assigned for **1a**, and those with the larger *transoid* coupling are for **1b**. The proposed amide conformation was confirmed by the NOESY experiment (Figure 16). The enhancement between δ 5.15 (1-N*H*) and 1.30 (H_3-15) of the *cisoid* conformer and that among δ 8.20 and (H-16) and δ 1.51 (H-2) of the *transiod* were observed and agreed well with the results for the analysis of the coupling constants. In addition, the parallel correlations between H-10α (δ 1.47) and H-12 (δ 0.80); and H-5 (δ 1.26) and H-7 (δ 1.45), of the *cisoid* conformer, and between the corresponding H-10α (δ 1.47) and H-

12 (δ0.80), and H-5 (δ1.26) and H-7 (δ1.40), of the *transoid* one, indicated the similar *endo* orientation of the isopropyl groups in both conformers. Notice that despite an approximation of 1:1 ratio for the *cisoid*/*transoid* conformation, slight predominating *cisoid* conformer can be observed (for examples, see the slight different integration among the resonances at δ8.02 [H-16] and 5.15 [1-N*H*] for the *cisoid* conformer and at δ8.20 [H-16] and 5.58 [1-N*H*] for the *transoid*). The conformational effects between two rotamers are to be discussed extensively in section 3.1.1.2, in which the predominating *cisoid* species is more prominent in compound **2**.

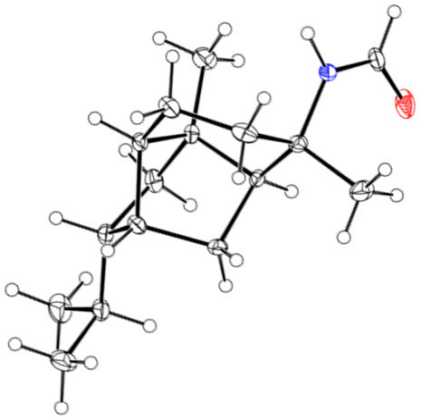


Figure 15. ORTEP drawing for the structure of **1** (*cisoid*).

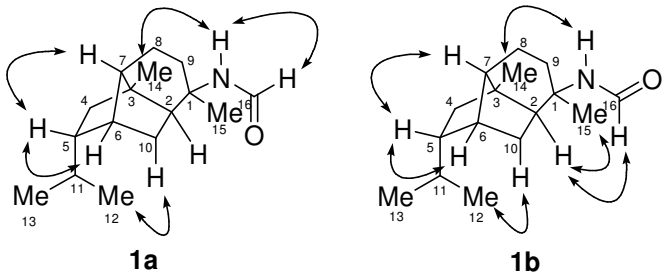


Figure 16. Dipolar couplings observed from the NOESY spectrum of **1** in the *cisoid* (**1a**) and *transoid* (**1b**) conformations.

3.1.1.2 2-Formamidopupukeanane (2)

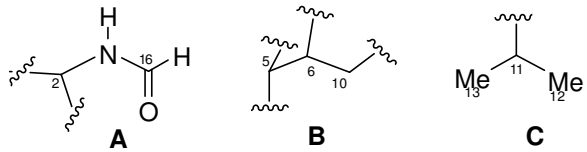
Compound **2** was obtained as a colorless solid. The molecular formula of $C_{16}H_{27}NO$ was deduced from HREIMS *m/z* 249.2087 $[M]^+$, and indicates that **2** is a constitutional isomer of **1**. The proposed molecular formula requires the unsaturation degree of 4, one of which is a carbonyl; hence, three rings are needed. Similar to **1** the IR spectrum of **2** showed the

characteristic absorption of a secondary amine and an amide carbonyl stretchings at 3300 and 1662 cm^{-1}, respectively. This is in agreement with the ^{13}C NMR spectrum, which displayed the resonances of formyl carbon at δ 161.1 ppm for the major conformer and at δ 164.7 ppm for the minor one. The UV spectrum shows the maximal absorption at λ 290 nm ($\log\varepsilon$ 2.08, MeOH).

Similar to **1**, compound **2** has two sets of NMR signals in a 2:1 ratio as a result of the rotating formamide moiety. The formamide group of the major conformer was observed at δ 8.25 (dd, *J* = 2.0, 0.5 Hz, H-16) and 5.43 (br s, 2-N*H*), and that of the minor one was at δ 7.90 (d, *J* = 11.9 Hz, H-16) and 5.76 (br s, 2-N*H*). The discussion hereafter is focusing on the major conformer in the same manner as that for **1**. The corresponding chemical shifts of the minor conformer are tabulated accordingly in Table 6.

The ^{13}C-NMR spectrum of **2** (Figure 20) displayed 16 carbons, including one carbonyl, two quarternary carbons, four methines, four methylenes, and four methyls. The ^{1}H NMR spectrum (Figure 19) of **2** exhibited two aliphatic methyl doublets (δ 0.82, d, *J* = 6.4 Hz, 6H, H_3-12 and H_3-13), and two aliphatic methyl singlets (δ 0.91, H_3-14; 0.76, H_3-15), one formyl proton (δ 8.25, dd, *J* = 2.0, 0.5 Hz, H-16), and one amide proton (δ 5.43, br d, *J* = 5.9 Hz). Except for an additional methine protons at δ 3.59 (d, *J* = 10.5 Hz, H-2), the remaining signals of **2** were all overlapped in the high field range (1.13-1.92 ppm).

The structure determination of **2** was conducted in the same manner to that of **1**. Interpretation of the $^{1}H,^{1}H$-COSY spectrum yielded three fragments; fragment **A**, from δ_H 8.25 (dd, *J* = 2.0, 0.5 Hz, H-16) through 5.43 (br d, *J* = 5.9 Hz, N*H*-2) to 3.59 (d, *J* = 10.5 Hz, H-2); fragment **B**, from δ_H 1.13 (m, H-5) through 2.04 (m, H-6) to 1.52 and 1.21 (m, H_2-10); and fragment **C**, from δ_H 1.45 (m, H-11) to 0.82 (d, *J* = 6.4 Hz, H_3-12 and H_3-13).



A **B** **C**

Connection among the three fragments was carried out by means of the HMBC correlation analysis. The crucial correlations from the HMBC spectrum include those from δ_H 3.59 (d, *J* = 10.5 Hz, H-2) to δ_C 161.1 (C-16), 49.1 (C- 4), 44.1 (C-7), 42.4 (C-3), 30.5 (C-1), 27.6 (C-10) and 25.4 (C-15), from δ_H 0.76 (s, H-15) to δ_C 62.4 (C-2), 33.8 (C-9), 30.5 (C-1) and 27.6

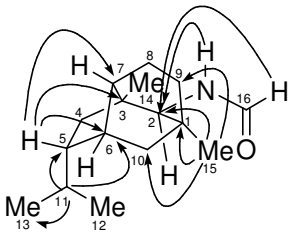
(C-10), from δ_H 1.13 (m, H-5) to δ_C 49.1 (C- 4), 42.4 (C-3), 38.8 (C-6) and 29.3 (C-11), from δ_H 1.45 (m, H-11) to δ_C 49.7 (C-5), 49.1 (C- 4), 38.8 (C-6), 21.6 (C-12) and 21.6 (C-13) (Figure 17). The formamide and isopropyl groups were placed on C-2 and C-5 also due to HMBC correlations from N*H* and H-16 to C-2, and from H-11 to C-5 and C-6, respectively. The structure of **2** was proposed to be 2-formamidopupukeanane. The compound was first reported as an intermediate through the structure determination of 2-isocyanopupukeanane without any spectroscopic description. Here, **2** was reported as a natural product for the first time with a complete detail on its spectroscopic data. The configuration of **2** as shown is drawn on the assumption of similar biological original to that of **1** (1*S**,2*S**,3*S**,5*S**,6*R**,7*R**).

Table 6. ^{1}H and ^{13}C NMR spectral data of **2** (500 MHz for ^{1}H, 125 MHz for ^{13}C; $CDCl_3$)

Position	δ_H (*J* in Hz)		δ_C	
	cisoid	*transoid*	*cisoid*	*transoid*
1	-	-	30.5 (C)	30.9
2	3.59 d (10.5)	2.73 d (11.0)	62.4 (CH)	68.6
3	-	-	42.4 (C)	43.2
4α	1.34 m	1.90 m	49.1 (CH_2)	49.5
4β	1.92 m	1.90 m	-	-
5	1.13 m	1.37 m	49.7 (CH)	49.6
6	2.04 m	2.04 m	38.8 (CH)	38.2
7	1.20 m	1.23 m	44.1 (CH)	44.0
8α	1.67 m	1.72 m	17.6 (CH_2)	17.4
8β	1.46 m	1.50 m	-	-
9α	1.20 m	1.30 m	33.8 (CH_2)	33.5
9β	1.16 m	1.30 m	-	-
10α	1.21 m	1.16 m	27.6 (CH_2)	27.5
10β	1.52 m	1.56 m	-	-
11	1.45 m	1.48 m	29.3 (CH)	29.4
12	0.82 d (6.4)	0.83 d (6.4)	21.6 (CH_3)	21.6
13	0.82 d (6.4)	0.83 d (6.4)	21.6 (CH_3)	21.6

Table 6. (cont.)

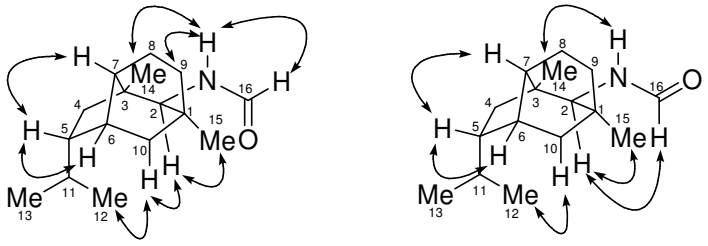
Position	δ_H (*J* in Hz)		δ_C	
	cisoid	*transoid*	*cisoid*	*transoid*
14	0.91 s	0.91 s	21.7 (CH_3)	21.7
15	0.76 s	0.77 s	25.4 (CH_3)	22.7
16	8.25 dd (2.0, 0.5)	7.90 d (11.9)	161.1 (C)	164.7
2-N*H*	5.43 br d (5.9)	5.76 m	-	-



2

Figure 17. Selected HMBC correlations of **2**

Also carried out in the same manner as that for **1** was the determination for the formamide geometry. The coupling constants of 2.0 Hz at δ8.25 and 11.9 Hz at δ7.90 indicated the *cisoid* and *transoid* conformation of the major and minor conformers, respectively. The NOESY spectrum, which showed dipolar couplings between H-14 and 2-N*H* of the major conformer, and between H-16 and H-2 of the minor conformer supported the previous analysis (Figure 18). The nOe enhancements among H-2 (δ3.59), H-10α (δ1.21), and H-12 (δ0.82); and between H-5 (δ1.13) and H-7 (δ1.20) of the major conformer, and those betweem H-10α (δ1.16) and H-12 (δ0.83), and between H-5 (δ1.37) and H-7 (δ1.23) of the minor conformer, simultaneously indicate the *endo* oreintation of the isopropyl group on C-5, and the *exo* orientation of the formamide moiety on C-2.



2a **2b**

Figure 18. Dipolar couplings observed from the NOESY spectrum of **2** in the *cisoid* (**2a**) and *transoid* (**2b**) conformations.

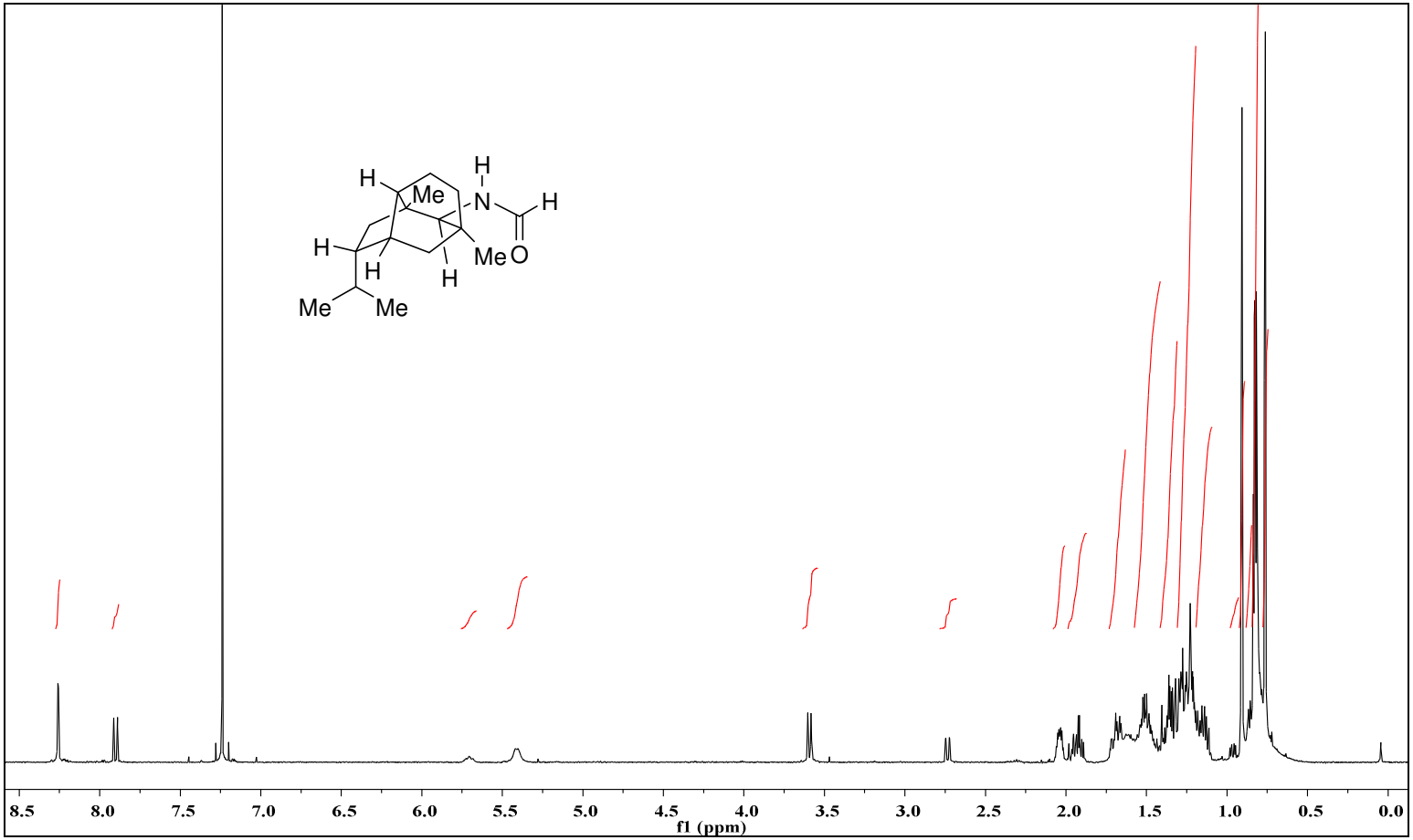


Figure 19. ^{1}H-NMR spectrum of **2** (500 MHz, $CDCl_3$)

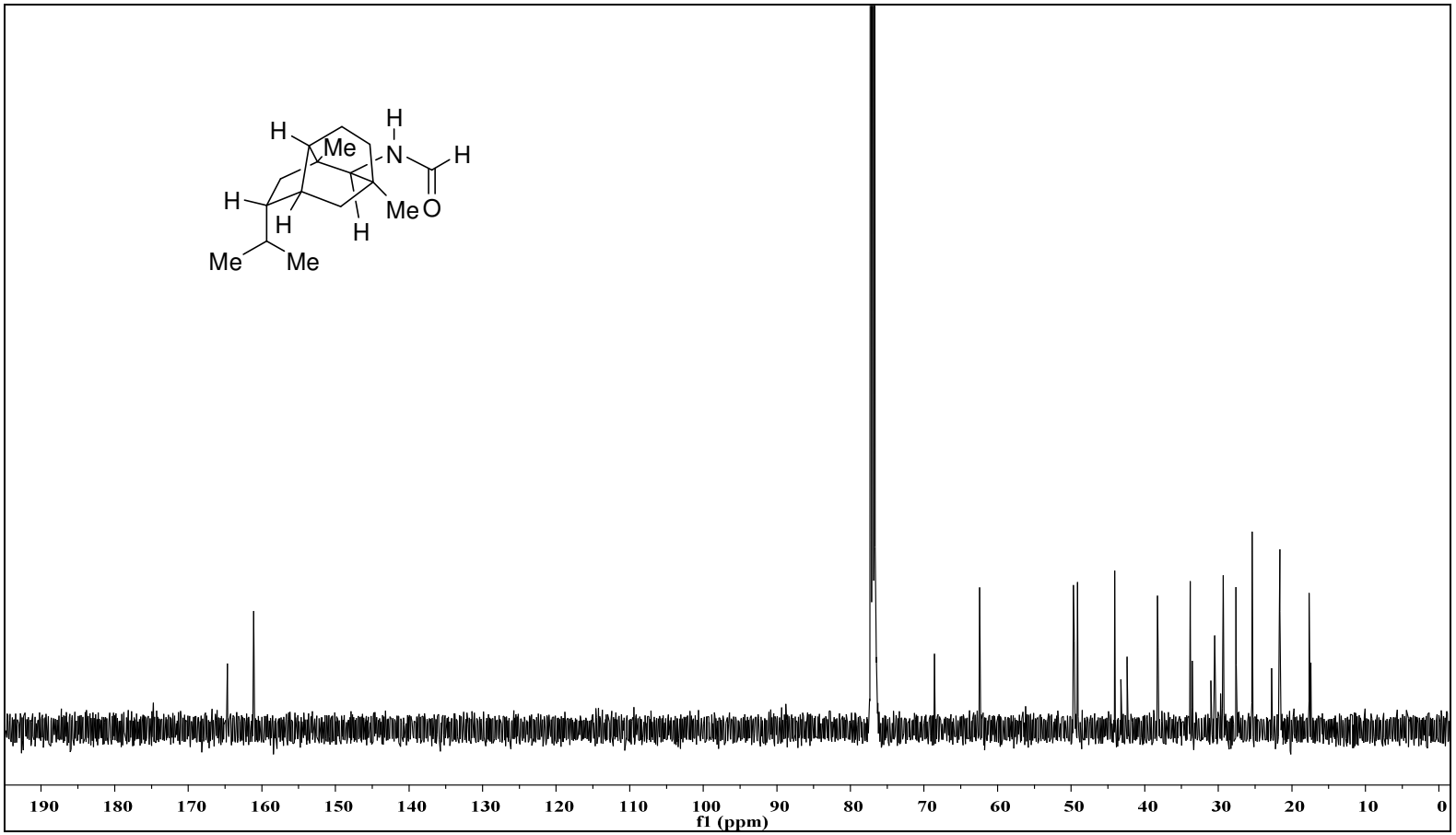


Figure 20. ^{13}C-NMR spectrum of **2** (125 MHz, $CDCl_3$)

3.2 Determination of the absolute configuration of 1

As stated in section 3.1.1.1, the x-ray crystallographic analysis unambiguously provided the relative configuration of compound **1**. Analysis of the coupling constants at formamide group also allowed the determination for the geometry of the formamide moiety of the two conformers, which was later confirmed by the NOESY experiment. The structures of **1** and **2** as depicted in sections 3.1.1.1 and 3.1.1.2 are arbitrarily drawn as referred to the structures of pupukeananes analogs previously reported, (for examples, see Burreson *et al*., 1975; Hagadone *et al*., 1979; Fusetani *et al*., 1990; Fusetani *et al*., 1991; Yasman *et al*., 2003). However, it is of interest to determine the absolute configuration of both compounds therefore to concur with the configuration of other related pupukeanane congeners.

Whereas there are a wide range of possible approaches to be employed, the limited amount of the isolated compounds (2.1 mg of **1**, 1.6 mg of **2**) forbade the chemical derivatization of either compound for the configurational analysis; hence, the non-destructive approach of ECD analysis was considered. The newly reported compound, compound **1**, was selected as the primary model for this part of investigation. The ECD spectra of **1** in MeOH (**a**) and $CHCl_3$ (**b**) were obtained (Figure 21). In non-polar solvent, **1** yields the first negative Cotton effect at λ 230 nm. In line with the UV absorption spectrum, a bathochromic shift toward λ 295 nm was observed upon changing the solvent to the polar protic solvent of MeOH.

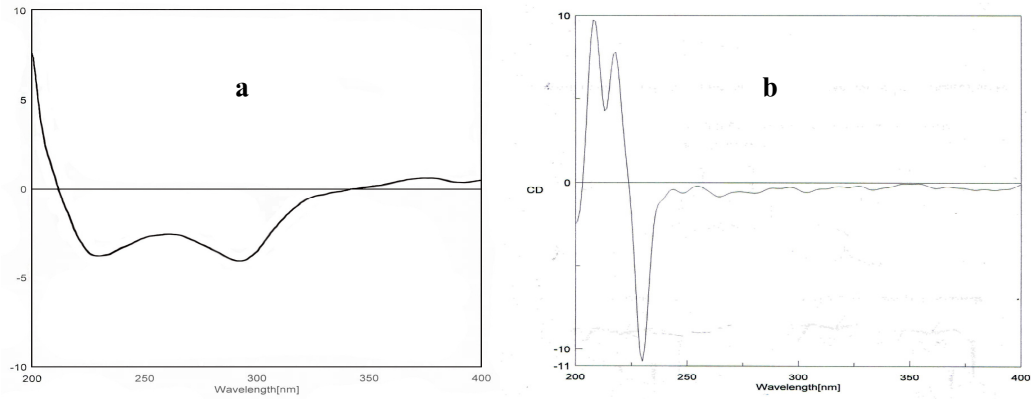


Figure 21. CD spectra of **1**; **a.** in MeOH, **b.** in $CHCl_3$

For a carbonyl compound possessing a chromophore adjacent to the chiral sphere, sector rule can generally be employed for the elucidation of its CD spectrum. However,

the relevant amide functionality of **1** protrudes and dangles outside the sphere of the structure. The bond between C-1 and the substituted nitrogen rotates freely; hence the octants in which the chiral sphere of the abeopupukeanane skeleton may reside cannot be resolved unambiguously (Figure 22). In addition, the two conformations that are caused by the rotatable formamide functionality further extend the complication. In fact, the thorough search has revealed that the dangling rotatable amide moiety similar to **1** has never been extensively explored for the chiroptical effects, and no closely related compounds are available for a direct comparison. Here, two approaches have been attempted in order to account for the CD spectrum of **1**. The first approach was to calculate the ECD spectra by means of the DFT analysis. The other is to synthesize a model with a close resemblance in the core skeleton and the hanging amide for a direct comparison between the ECD spectra of the synthesized models and the isolated compound.

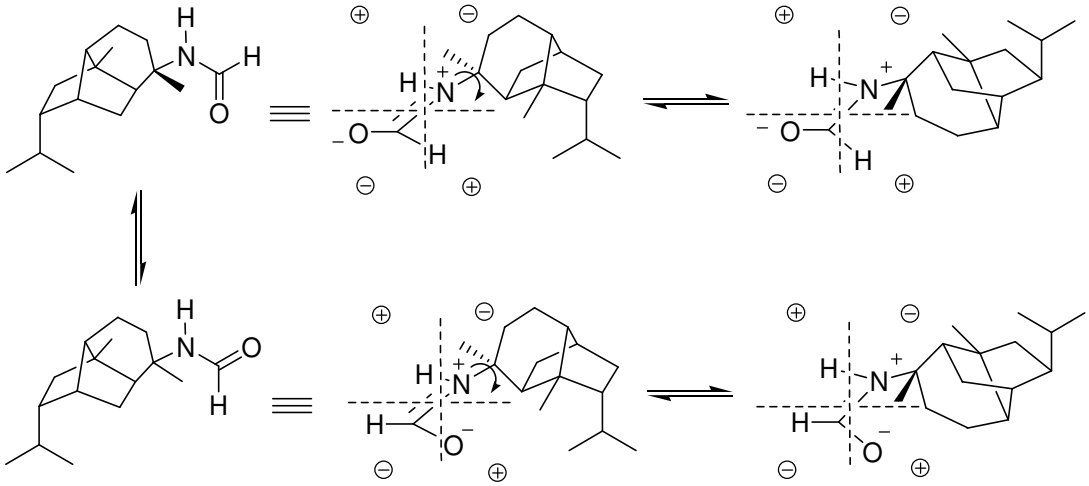


Figure 22. The conformations of **1** based on sector rule

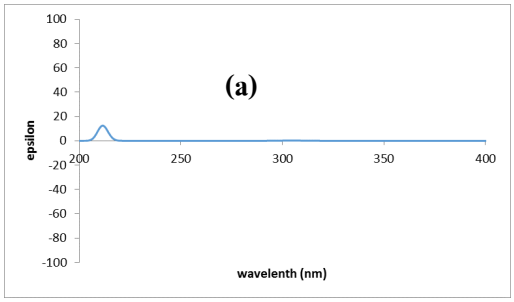
3.2.1 DFT calculation

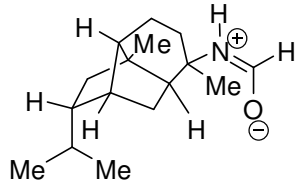
The preferred conformations of compound **1** are optimized using Gaussian 03 in the gas phase at the B3LYP/6-31G(d). The possibility of the conformational changes and the variation in the electronic states (either as a resonance amidate or as a zwitterion) in the solution phases has been taken into the consideration, and the conformational minima of 12 possible conformations, four of which unaffected by H-bond from MeOH (Figures 23 a-d), and eight others in MeOH (Figures 23 e-l), for one possible enantiomer have been prepared. These include

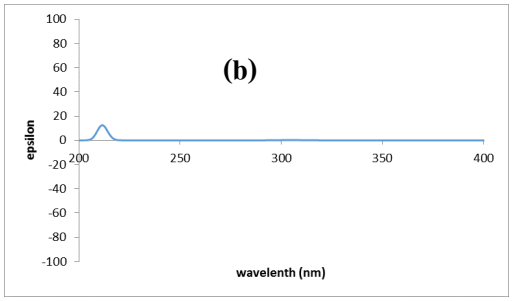
the possibility of *cisoid* and *transoid* conformations in both solvents, and with H-bond toward both H-bond acceptors and donors on the formamide moiety when prepared as a solution in MeOH.

CD spectrum of each optimized conformation was calculated (Figure 23). Unfortunately, none are consistent with the observed ECD spectra, either in the $CHCl_3$ or in MeOH. The inability of DFT calculation to predict the ECD spectra of the formamide, albeit disappointing, was not unexpected. With the rotation about the C-N bond on C-1, the conformations of the formamide as depicted in Figure 22 suggested that there might be more than one predominant species that may strongly influence the chiroptical phenomenon. Also, it is difficult to deduce the real existence of the proposed conformation, therefore further complicate the estimation for the percentage of the conformations that may exist in the solution. Therefore, presumption based on the possible average spectra from the calculation cannot be carried out.

The results from DFT calculation, however, agree well with the bathochromic shift caused by H-bonds between either N or O H-bond acceptor of the formamide and MeOH. The Cotton effects of all the conformation proposed to involve H-bond are observed as far as 300 nm, similar to the observed ECD spectra of **1** in MeOH (Figures 23 – e-l).







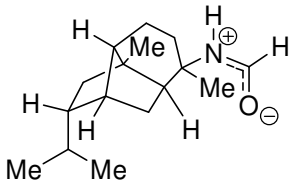
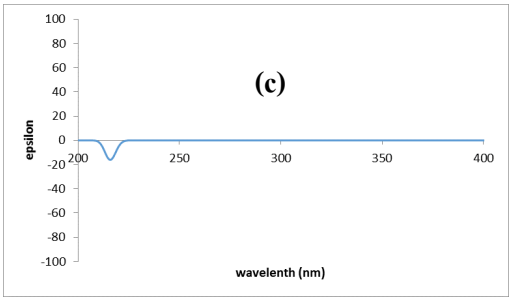
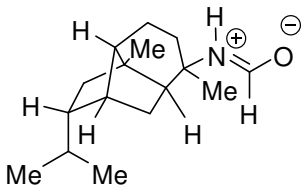
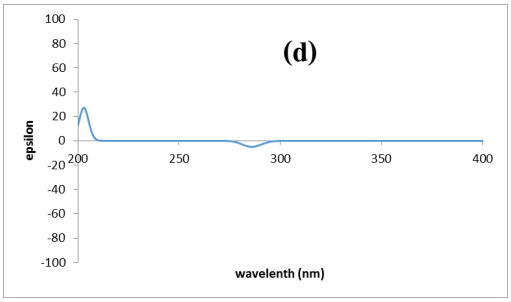
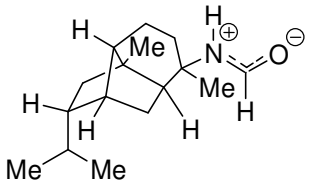


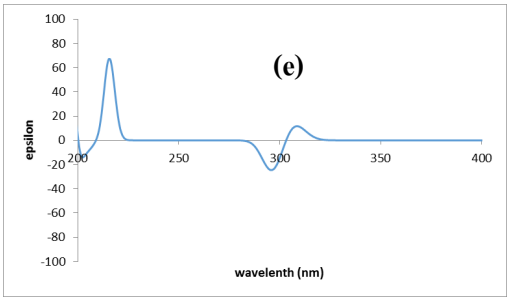
Figure 23. Calculated ECD spectra of **1**; (**a** and **b**) zwitterion of *cisoid* in $CHCl_3$,(**c** and **d**) zwitterion of *transoid* in $CHCl_3$, (**e** and **g**) O H-bond of *cisoid* in MeOH, (**f** and **h**) N H-bond of *cisoid* in MeOH, (**i** and **k**) O H-bond of *transoid* in MeOH, (**j** and **l**) N H-bond of *transoid* in MeOH.

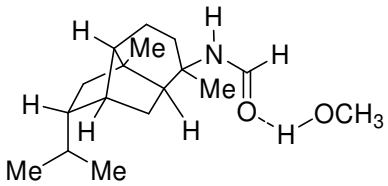


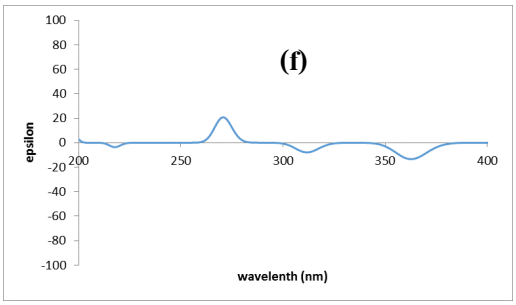


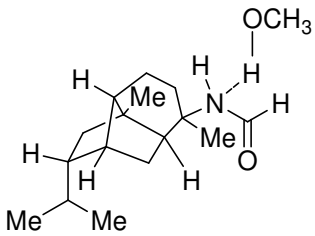


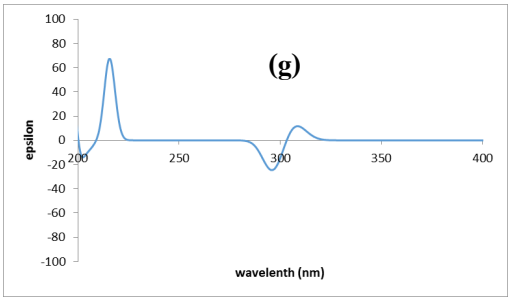












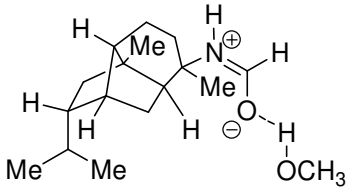
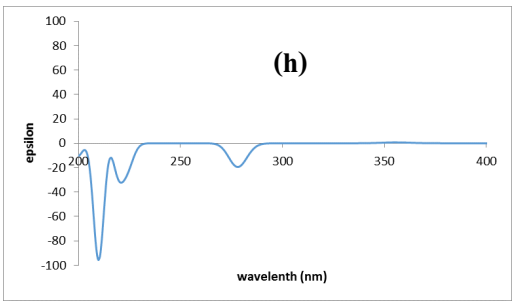
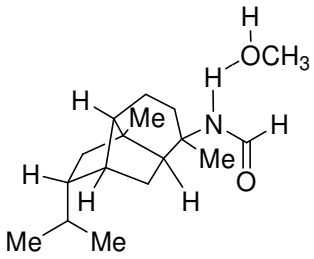
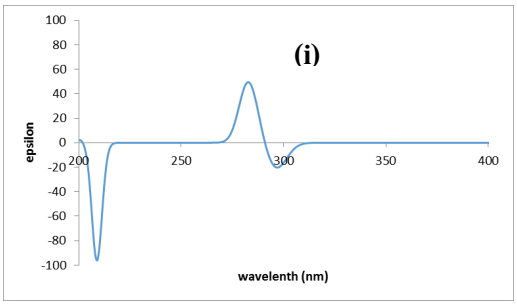
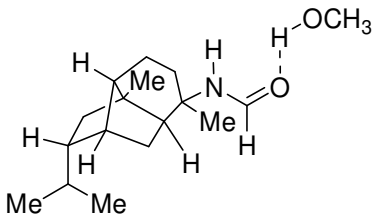


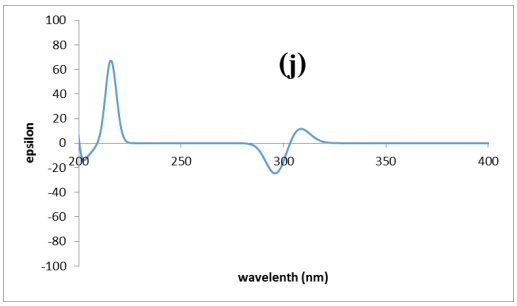
Figure 23 (cont.)

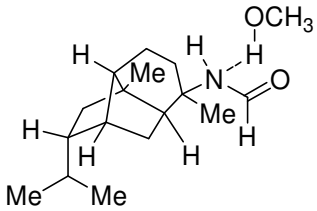


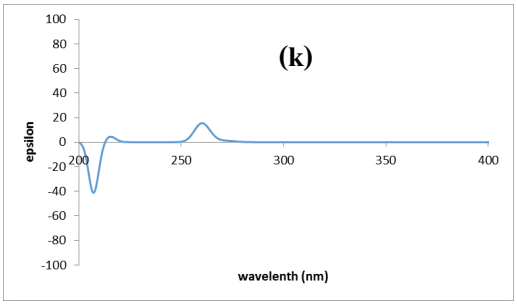


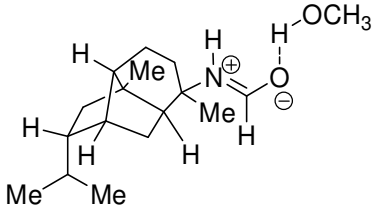


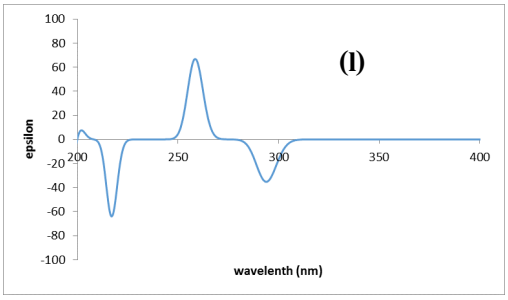












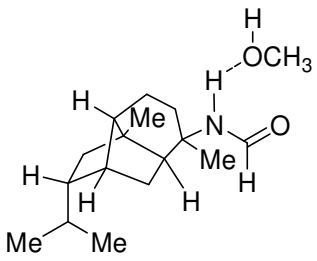
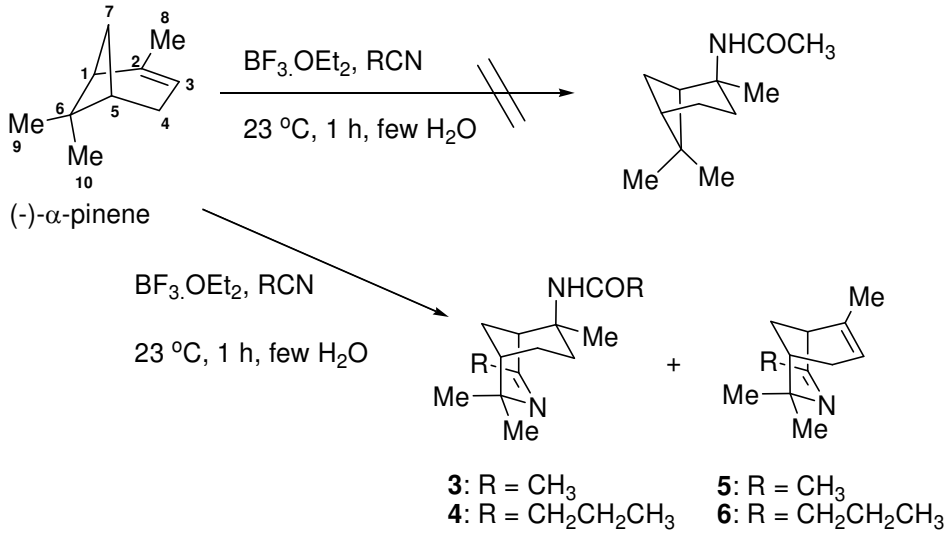


Figure 23. (cont.)

3.2.2 The model structures of amide pendants

For any compounds possessing the structures that have never been thoroughly studied for the ECD phenomenon, it has been suggested that the ECD spectra of model compounds that have closely similar chemical structures to such target molecules may also allow the direct comparison, hence leading to the interpretation of the ECD spectra. However, upon thorough search for similar chemical entities, it appears that the compounds with bridged structures that possess an amide in such a dangling orientation are rare, and the ECD spectra of such a structural genre are not available. Here, syntheses of bridged bicyclic compounds with amide pendant have been attempted.

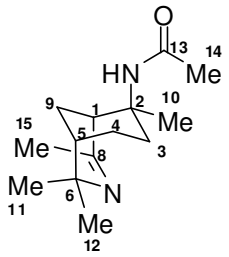
(-)-α-Pinene was selected as a starting material. Amidation using alkyl nitriles in $HBF_4.OEt_2$ onto an alkene (Subba *et al*., 2010) were reported to yield the corresponding amides in a smooth manner (Scheme 2). Two nitriles, including MeCN and *n*-PrCN in $HBF_4 \cdot OEt_2$ were attempted. However, instead of the expected amides, surprisingly and disappointingly, ring expansion products were abtained (Scheme 2).



Scheme 2. Amidation of α-pinene

The structural elucidations of all the modified models are as followed. Compound **3** was prepared from an amidation of (-)-α-pinene with acetonitrile in the presence of $HBF_4 \cdot OEt_2$. The molecular formula at $C_{14}H_{24}N_2O$ was proposed according to the molecular ion

peak at *m/z* 236.1880 $[M]^+$ (calcd for $C_{14}H_{24}N_2O$ 236.1889), hence additional units of $C_4H_8N_2O$ were added onto the structure of pinene starting material. These include a methyl imine and an acetamido groups as indicated by the spin systems of δ_C 166.9 (C-8) and δ_H 2.01 (s, H_3-15) for the imine; and at δ_C 167.7 (C-13) and 24.5 (C-14) and at δ_H 5.54 (br s, N*H*-2) and 1.92 (s, H_3-14) for the acetamido moiety (Tables 7 and 8). The remaining proton and carbon resonances of **3** almost resembled those of pinene, and the structure determination of all the high-field nuclei was carried out by means of HBMC spectral analysis to show that all are conserved from the core structure of pinine. Specifically for the newly substituted groups, the acetamido moiety were at C-2 based on the correlations from δ_H 5.54 br s (N*H*) to δ_C 169.7 (C-13), 55.6 (C-2), 40.2 (C-1) and 33.1 (C-3), and that of the imine were at C-8 based on the correlations from δ_H 3.12 (br d, 1.5; H-1) to δ_C 166.9 (C-8), 55.6 (C-2), 33.8 (C-5), 29.7 (C-15) and 24.0 (C-9) and δ_H 2.01 (s, H_3-15) to δ_C 166.9 (C-8). The structure of **3** was therefore proposed to be 8-acetamido-2,4,4,8-tetramethyl-3-azabicyclo[3.3.1]-non-2-ene.



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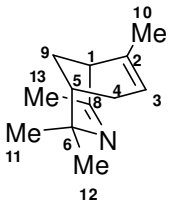
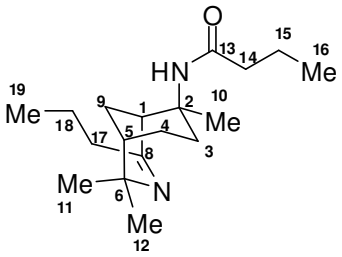


5

In addition to compound **3**, side reaction from the amidation of (-)-α-pinene with acetonitrile in the presence of $HBF_4 \cdot OEt_2$ was obtained. Compound **5** has a molecular formula of $C_{12}H_{19}N$ according to the mass at *m/z* 177.1517 in the HREIMS spectrum (calcd for of $C_{12}H_{19}N$, 177.2859, $[M]^+$). Compared with **3**, **5** has an additional unit of C_2H_3N, consistent with a methyl imine moiety. This was observed at δ_C 170.5 (C-8) and at δ_H 2.08 (s, H_3-13) (Tables 7 and 8). The structure of **5** was therefore proposed to be 2,6,6,8-tetramethyl-7-azabicyclo[3.3.1]-non-2-ene.

Amidation of (-)-α-pinene with *n*-buthylnitrile in the presence of $HBF_4 \cdot OEt_2$ yielded compound **4**. The molecular formula of $C_{18}H_{32}N_2O$ was proposed according to the mass of 292.2509 $[M]^+$ in the HREIMS (calcd for $C_{18}H_{32}N_2O$, 292.2515), hence the units with $C_8H_{16}N_2O$

were added onto the starting structure of pinene. In a parallel manner to that of **3**, the two added units of **4** was a propyl imine and a butyroamide moiety. The propyl imine moiety was observed at δ_C 178.3 (C-8) and at δ_H 2.40 (td, 8.0, 1.5, H_2-17), 1.50 (m, H_2-18) and 0.91 (t, 7.5, H_3-19) in the NMR spectra (Tables 7 and 8), whereas the butyroamide group was at δ_C 173.4 (C-13) and at δ_H 2.14 (td, 7.5, 3.0, H_2-14), 1.61 (m, H_2-15) and 0.91 (t, 7.5, H_3-16). The determination of the remaining resonance was carried out in the same manner as that for **3**, and the structure of **4** was proposed to be 8-propylamido-2-propyl-4,4,8-trimethyl-3-azabicyclo[3.3.1]-non-2-ene.



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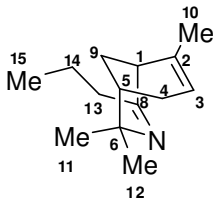


6

Similar to compound **5**, compound **6** was also obtained as a side product of **4** during the amidation of (-)-α-pinene with *n*-buthylnitrile in the presence of $HBF_4 \cdot OEt_2$. It has a molecular formula of $C_{14}H_{23}N$ based on HREI mass spectra (*m/z* 205.18305 $[M]^+$, calcd for $C_{14}H_{23}N$, 205.18315). The propylimine moiety, transformed in the same manner as that for **5** (Tables 7 and 8) was observed at δ_C 190.5 (C-8) and at δ_H 2.88 (m, H-13), 2.60 (m, H-13), 2.04 (m, H-14), 1.89 (m, H-14) and 1.01 (t, 7.5, H_3-15). The elucidation for the remaining resonances of **6** was executed in the same manner as that for **5**. The structure of **6** was therefore proposed to be 2,6,6-trimethyl-8-propyl-3-azabicyclo[3.3.1]-non-2-ene.

Table 7. ^{1}H NMR spectral data of **3**-**6** and (-)-α-pinene (500 MHz; $CDCl_3$) (δ in ppm, *J* in Hz)

Position	**3**	**4**	**5**	**6**	(-)-α-pinene
1	3.12 br d (1.5)	3.59 s	2.56 m	3.15 br s	1.96 m
2	-	-	-	-	-
3ax	1.33 td (14.5, 5.0)	1.32 m	5.35 m	5.64 m	5.20 m
eq	1.44 dm (14.5)	1.56 m			
4ax	1.52 tt (14.5, 4.5)	1.66 m	2.22 dm (17.5)	2.32 m	2.21 m
eq	1.74 dm (14.5)	1.78 m	2.14 dm (17.5)	2.40 m	2.15 m
5	1.56 m	1.67 m	1.86 m	2.13 m	2.03 m
6	-	-	-	-	-
7	-	-	-	-	1.16 m
					2.36 m
8	-		-	-	1.26 s
9ax	1.65 ddd (13.5, 6.0, 3.0)	1.68 m	1.86 m	1.79 m	0.83 s
eq	1.70 ddd (13.5, 3.0, 1.5)	1.83 dt (13.0, 2.5)	1.56 ddd (13.5, 3.5, 2.5)	2.02 m	
10	1.38 s	1.39 s	1.78 dd (4.0, 2.0)	1.82 dd (4.0, 2.5)	1.64 m
11	1.19 s	1.34 s	1.21 s	1.50 s	
12	1.07 s	1.21 s	1.15 s	1.44 s	

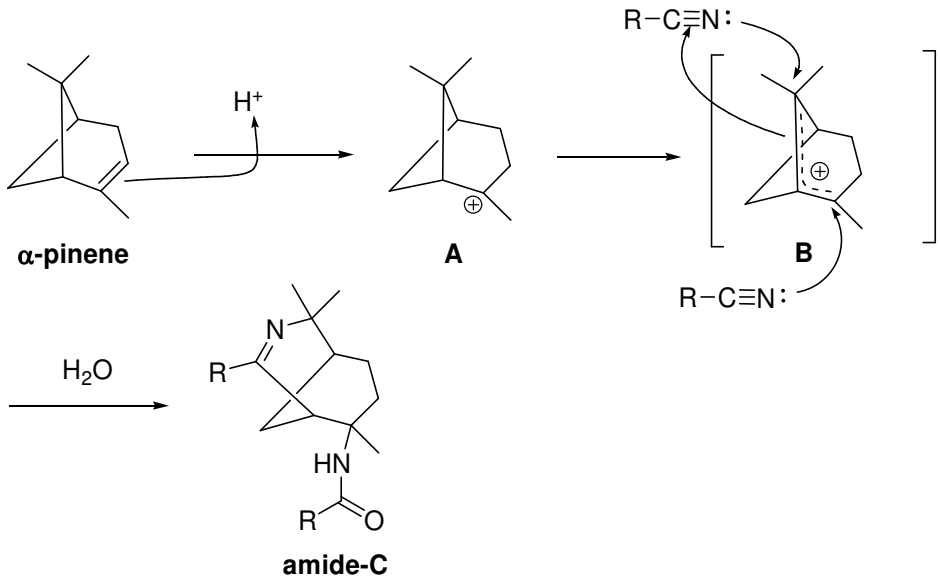
Table 7. (cont.)

Position	**3**	**4**	**5**	**6**	(-)-α-pinene
13	-	-	2.08 s	2.60 m	
				2.88 m	
14	1.92 s	2.14 td (7.5, 3.0)		1.89 m	
				2.04 m	
15	2.01 s	1.61 m		1.01 t (7.5)	
16		0.91 t (7.5)			
17		2.40 td (8.0, 1.5)			
18		1.50 m			
19		0.91 t (7.5)			
N*H*	5.54 br s	5.72 s			

Table 8. ^{13}C NMR spectral data of **3**-**6** and (-)-α-pinene (125 MHz; $CDCl_3$)

Position	**3**	**4**	**5**	**6**	(-)-α-pinene
1	40.2 (CH)	38.7 (CH)	40.3 (CH)	38.7 (CH)	47.0 (CH)
2	55.6 (C)	55.7 (C)	133.8 (C)	130.7 (C)	144.5 (C)
3	33.1 (CH_2)	32. (CH_2)	122.9 (CH)	126.7 (CH)	116.0 (CH)
4	24.5 (CH_2)	24.0 (CH_2)	28.6 (CH_2)	28.2 (CH_2)	31.3 (CH_2)
5	33.8 (CH)	33.8 (CH)	33.2 (CH)	33.3 (CH)	40.7 (CH)
6	57.9 (C)	59.3 (C)	58.9 (C)	62.0 (C)	38.0 (C)
7	-	-	-	-	31.5 (CH_2)
8	166.9 (C)	178.3 (C)	170.5 (C)	190.5 (C)	26.3 (CH_3)
9	24.0 (CH_2)	23.5 (CH_2)	25.0 (CH_2)	23.4 (CH_2)	20.8 (CH_3)
10	27.2 (CH_3)	26.8 (CH_3)	23.7 (CH_3)	23.2 (CH_3)	23.0 (CH_3)
11	26.3 (CH_3)	25.0 (CH_3)	31.2 (CH_3)	29.4 (CH_3)	
12	31.6 (CH_3)	30.9 (CH_3)	27.4 (CH_3)	24.9 (CH_3)	
13	169.7 (C)	173.4 (C)	27.7 (CH_3)	39.7 (CH_2)	
14	24.5 (CH_3)	39.0 (CH_2)		20.1 (CH_2)	
15	29.7 (CH_3)	19.0 (CH_2)		13.4 (CH_3)	
16		13.6 (CH_3)			
17		43.2 (CH_2)			
18		21.8 (CH_2)			
19		13.6 (CH_3)			

It was surprising to find that, despite the smooth reactions, the expected amides, i.e. singly amidation onto C-2 of pinene, were not obtained. Instead, along with amidation as seen in **3** and **4**, imination-ring expansion took place. The mechanism of imination-ring expansion (Scheme 3) is proposed here to be driven by the ring strain of the cyclobutane moiety within the bicyclic skeleton of pinene. Instead of regiospecific protonation onto C-2, such a ring strain led to an allyl carbocation, which facilitate the ring opening upon capturing the nucleophilic nitrogens of either nitrile. The presence of minor imination by-products in fact indicated that relieving the ring strain was the driving force, and presumably took place prior to the amidation.



Scheme 3. Mechanism of the amidation-ring expansion of α-pinene

Compounds **3** and **4** were subjected to ECD spectral measurement (Figures 24). The positive first Cotton effect was observed at λ 245 nm (in MeOH) ($\Delta\varepsilon$ 8.9 and 8.7) and at λ 248 and 230 nm (in $CHCl_3$); ($\Delta\varepsilon$ -8.8 and 9.3) for each compound. Interestingly, unlike the previously trialed formamides **1** and **2**, the ECD spectra of **3** and **4** were not influenced by the solvent effect; i.e., neither compound exhibited bathochromic shifts that might be caused by H-bond from MeOH (Figures 21 and 24). This strongly indicated that the chiroptical properties that lead to the ECD spectra of **3** and **4** were governed by the *n*-π^* transition of the imine moiety. The dangling amides seemingly exerted very little influences, and were not able to be applicable to the formamide group of **1**.

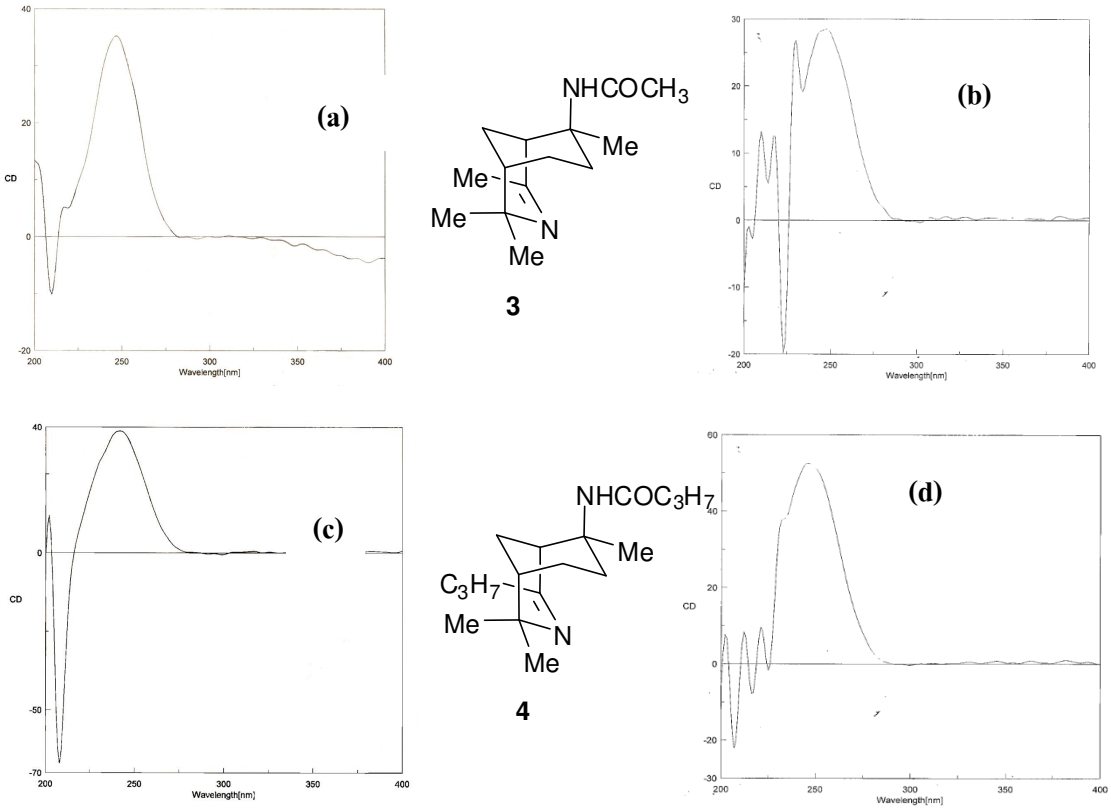


Figure 24. The CD spectra of **3** in MeOH (**a**) and in $CHCl_3$ (**b**); and **4** in MeOH (**c**) and in $CHCl_3$ (**d**)

3.3 The biogenetic origin

The biogenetic origin of the pupukeanane skeleton has been proposed to arise from amorphene (Karuso *et al*., 1989) (Scheme 4). From the first 1⟶6 cyclization of amorphene, a 4(2⟶3) shift would lead to the pupukeananes, whereas an 8(7⟶3) shift yields the neopupukeanane series. The 1,3-hydride shift followed by a 2(1⟶9) shift on the pupukeanane cation intermediate would result in the allopupukeanane structure. A 10(1⟶2) shift on the pupukeanane cation intermediate provides the abeopupukeanane structure. The CN^- salt attract to the pupukeanane cation and the addition of water to C isocyanide cation followed by keto-enol form tautomerism give rise to formamide functionality in abeopupukeanane (**1**) and pupukeanane (**2**).

As for the nitrogenated functionalities, it has been demonstrated that the closely formamide functionality in sponge-derived sesqui- and diterpenes was resulted from the isonitrile (Hagadone *et al*., 1984; Karuso and Scheuer, 1989a; Chang and Scheuer, 1990). However, unlike

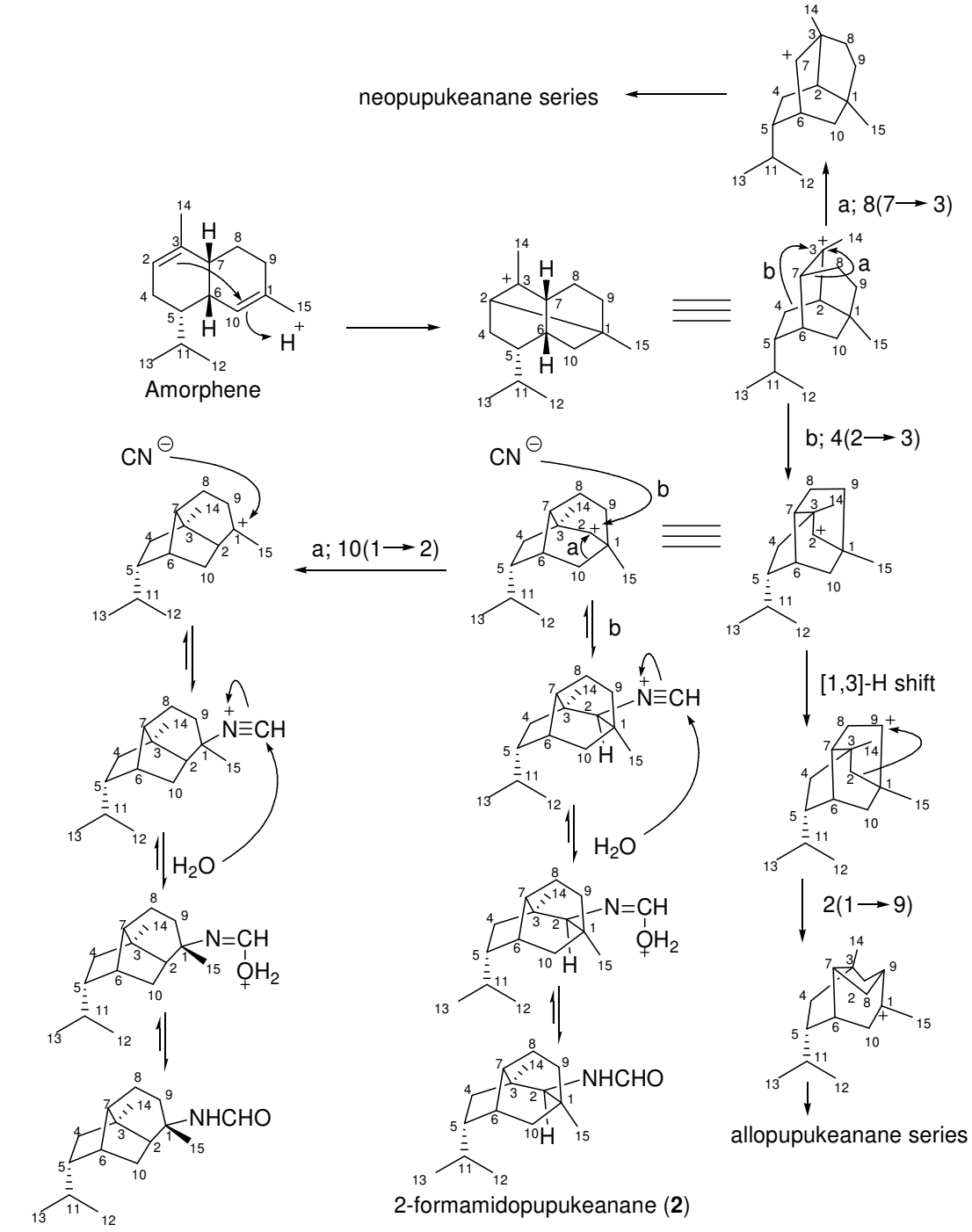
most nitrogenous natural products, including the majority of alkaloids and amide derivatives, of which nitrogen atoms come from amino acids, the source of the isonitrile in the pupukeananes and related marine-derived terpenes is the inorganic cyanide ion (Chang and Scheuer, 1990). For example, it has been demonstrated that the cyanide ion is the nitrogen source of 2-isocyanopupukeanane in the sponge Ciocalypta sp. (Hagadone *et al*., 1984; Karuso and Scheuer, 1989a; Chang and Scheuer, 1990). Similar results were reported with the isocyanoterpenes in sponges of other genera, including Amphimedon, Acanthella, and Axinyssa (Garson, 1986; Karuso and Scheuer, 1989a; Chang and Scheuer, 1990; Dumdei *et al*., 1997; Simpson and Garson, 1998; Simpson and Garson, 1999). It is proposed here that the formamide moiety in **1** and **2** may come from the isonitrile in a route similar to that of other pupukeananes and related sponge-derived terpenes, i.e., presumably incorporated as a cyanide ion by the sponges prior to being consumed by the mollusk.

3.4 Antiproliferative activity

Compounds **1** and **2** were subjected to antiproliferative activity determination using SRB assay (Skehan *et al*., 1990) and targeting HeLa, MCF-7, KB, and HT-29 cancer cell lines. Both compounds showed good activities particularly against HeLa cell lines, and a good to moderate potency against MCF-7 and KB (Table 9). The compounds were virtually inactive against normal fibroblast cells, inhibit at 65% and 25%, at 20 μM, respectively.

Table 9. Cytotoxic activity of compounds **1** and **2**

cytotoxic activity	**Samples (IC_{50} in μM ±SD)**		
	1	**2**	**Camptothecin**
HeLa	0.13 ± 0.012	0.07 ± 0.008	0.13 ± 0.003
MCF-7	0.65 ± 0.29	8.2 ± 0.29	0.0033 ± 0.0011
KB	2.4 ± 0.32	1.2 ± 0.10	0.02 ± 0.003
HT-29	6.8 ± 0.23	35% (observed at 20 μM)	0.0007 ± 0.0001
human gingival fibroblast % inch at 20 μM	-	-	-



Scheme 4. Proposed biogenetic origin of pupukeanane, neopupukeanane, and abeopupukeanane skeletons (atom numbering after that given for pupukeanane skeleton)

CHAPTER 4

CONCLUSION

The chemical investigation of the nudibranch *P. coelestis* Bergh lead to the isolation of two pupukeanane-type sesquiterpenes. Among these, compound **1**, 1-formamido-10(1⟶2)-abeopupukeanane, was a bridged tricyclic sesquiterpene with an unprecedented rearrangement of the tricyclo[4.4.0.$0^{2,8}$]decane skeleton. Compound **2**, 2-formamidopupukeanane, was previously reported as an intermediate for the structure determination of 2-isocyanopupukeanane. The compound was reported here as a natural product along with the complete spectroscopic data for the first time The investigation of the absolute configuration for the dangling and highly flexible formamide has been attempted. Unfortunately, the approaches by way of ECD spectral analysis were not completely accomplished. However, a suitable model with closely related tricyclic structures, may allow a direct comparison, hence open up an opportunity for the configuration determination of rotatable amides.

Many pupukeanane sesquiterpenes including **1** and **2** have been isolated from the nudibranch *P. coelestis* Bergh and related species. These results suggesting that the terpene origin might produce from sponge prays because these terpenes were reported from some sponges (Simpson and Garson 2004; Gulavita *et al*., 1986). Following the formamide functionality on pupukeanane has led to summarize a chemical relation between sea slug predators and sponge prays.

The isolated compounds were tested for their cytotoxicities against cancer cell lines. Both compounds showed antiproliferative activity when targeting HeLa, MCF-7, KB, and HT-29 cancer cell lines. The IC_{50} (±SD) values of **1** against HeLa, MCF-7, KB, and HT-29 cells were 0.13(0.012), 0.65(0.29), 2.4(0.32), and 6.8(0.23) μM, respectively, and those of **2** against HeLa, MCF-7, and KB were 0.07(0.008), 8.2(0.29), and 1.2(0.10) μM. As for the HT-29 cells, 35% inhibition was observed at 20 μM of **2**. Both compounds weakly inhibited the proliferation of human gingival fibroblast cells (65% and 25% inhibition, each at 20 μM, respectively).

Overall, this work has demonstrated that Thai marine organisms are among the potential sources of chemotherapeutic agents that may be useful in drug development. The further

exploration will lead to the compounds with greater efficacy and specificity for the treatment of tuberculosis, as well as other infectious and cancerous diseases. The pharmaceutical potentials of marine bioresources hopefully will lead to the national policy for a better and more sustainable utilization of bioresources, as well as to a strong and positive impact on pharmaceutical research and development both in the governmental and public sectors in Thailand.

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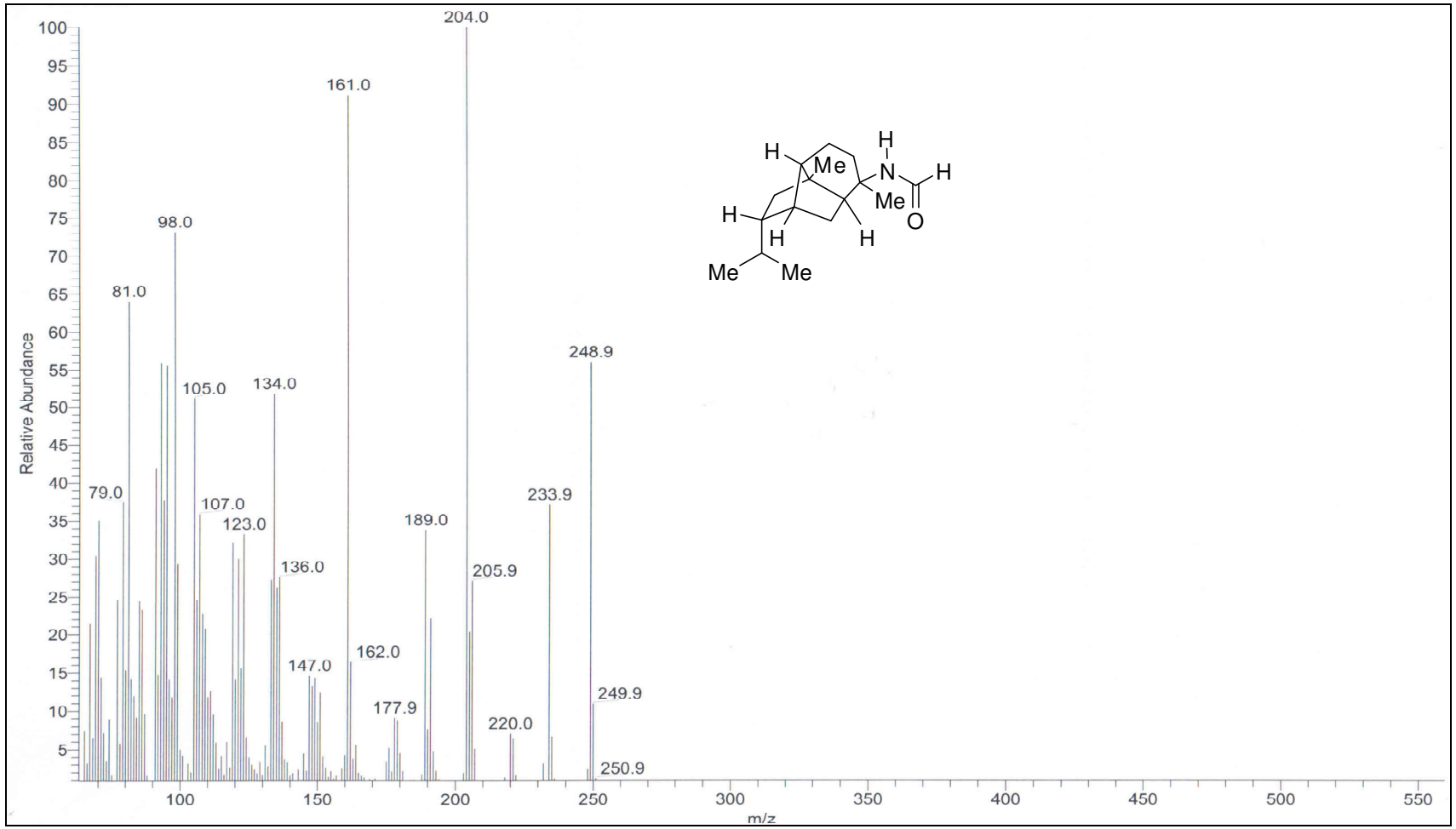
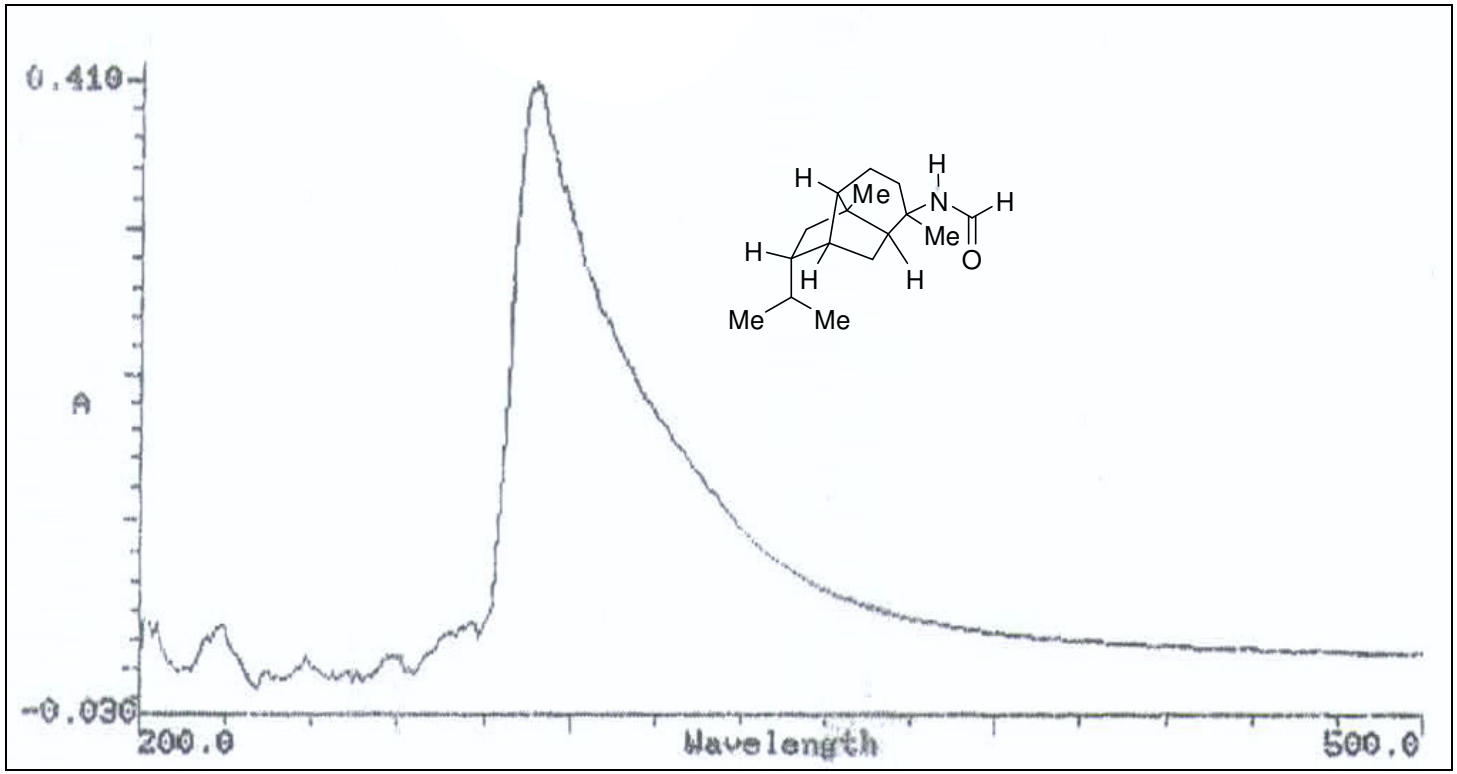
APPENDIX



EIMS spectrum of **1**



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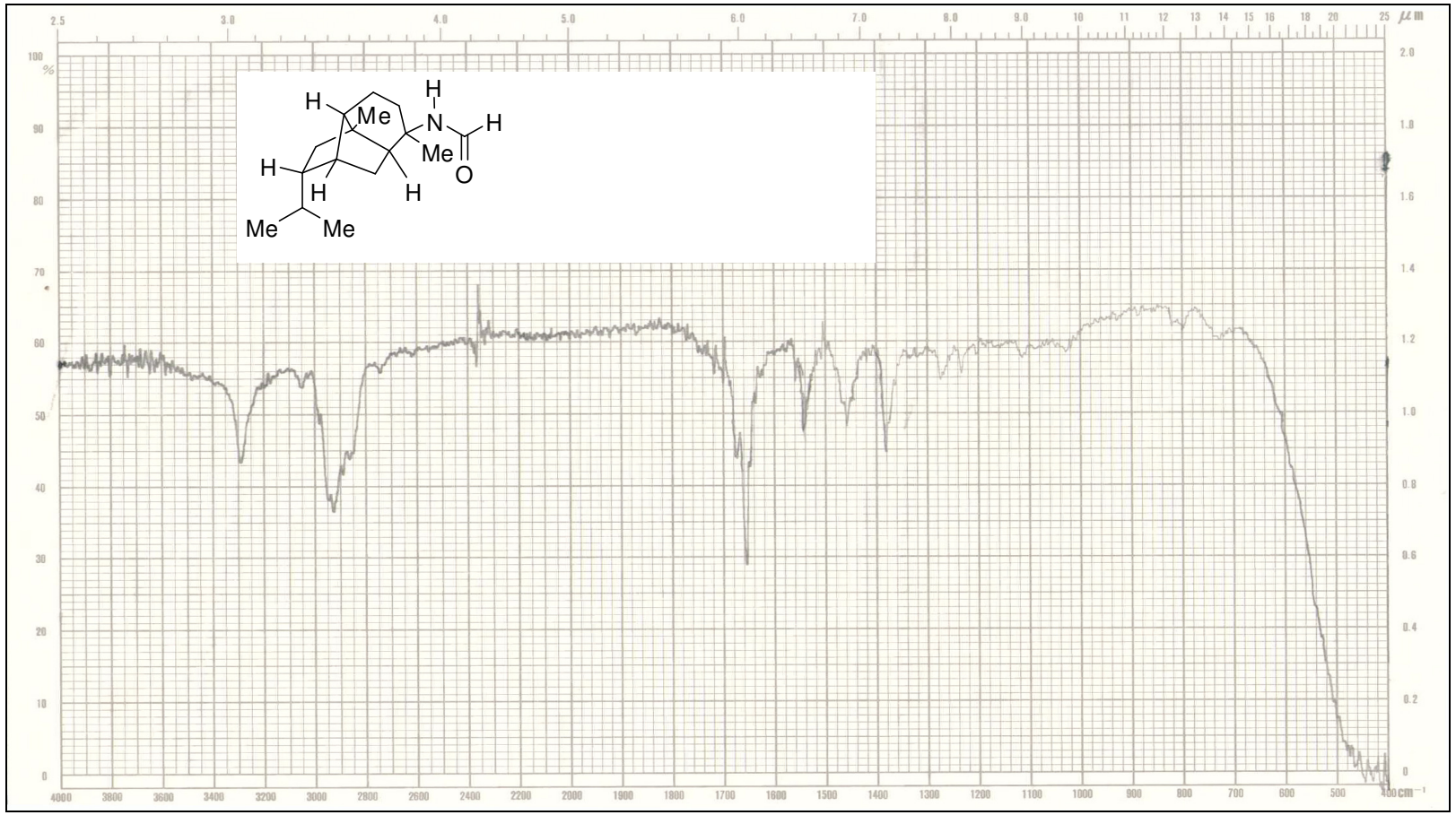

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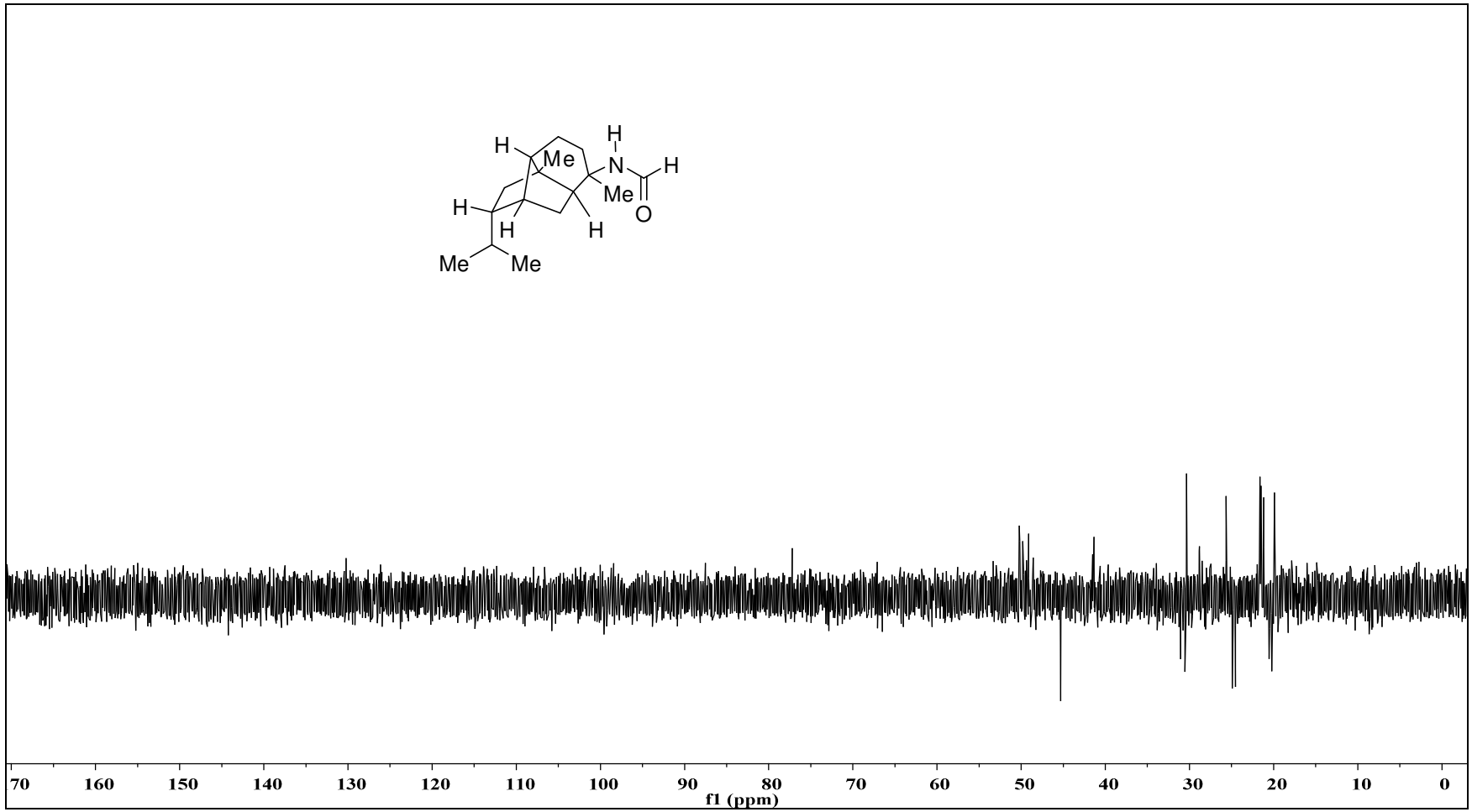

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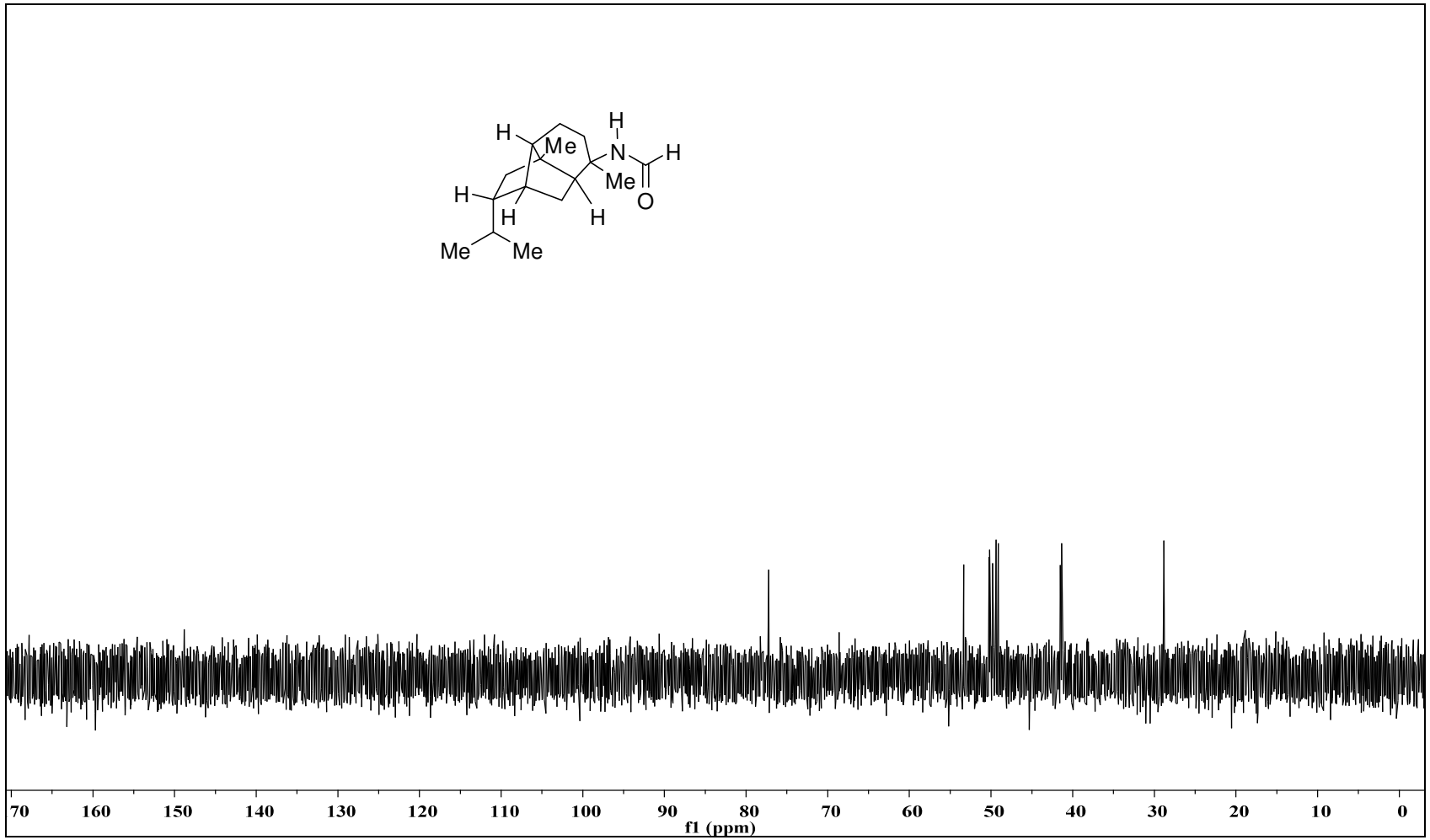

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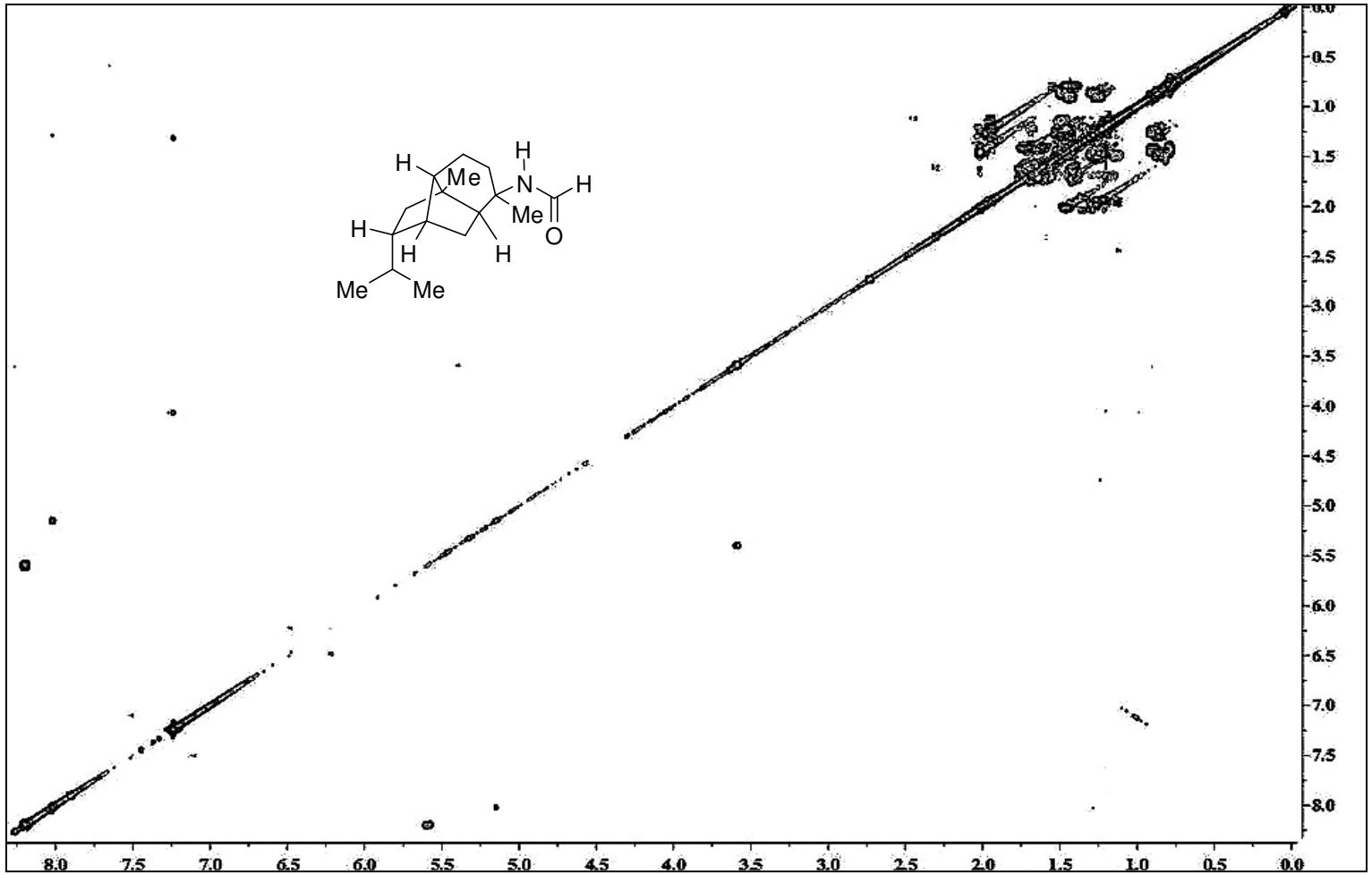
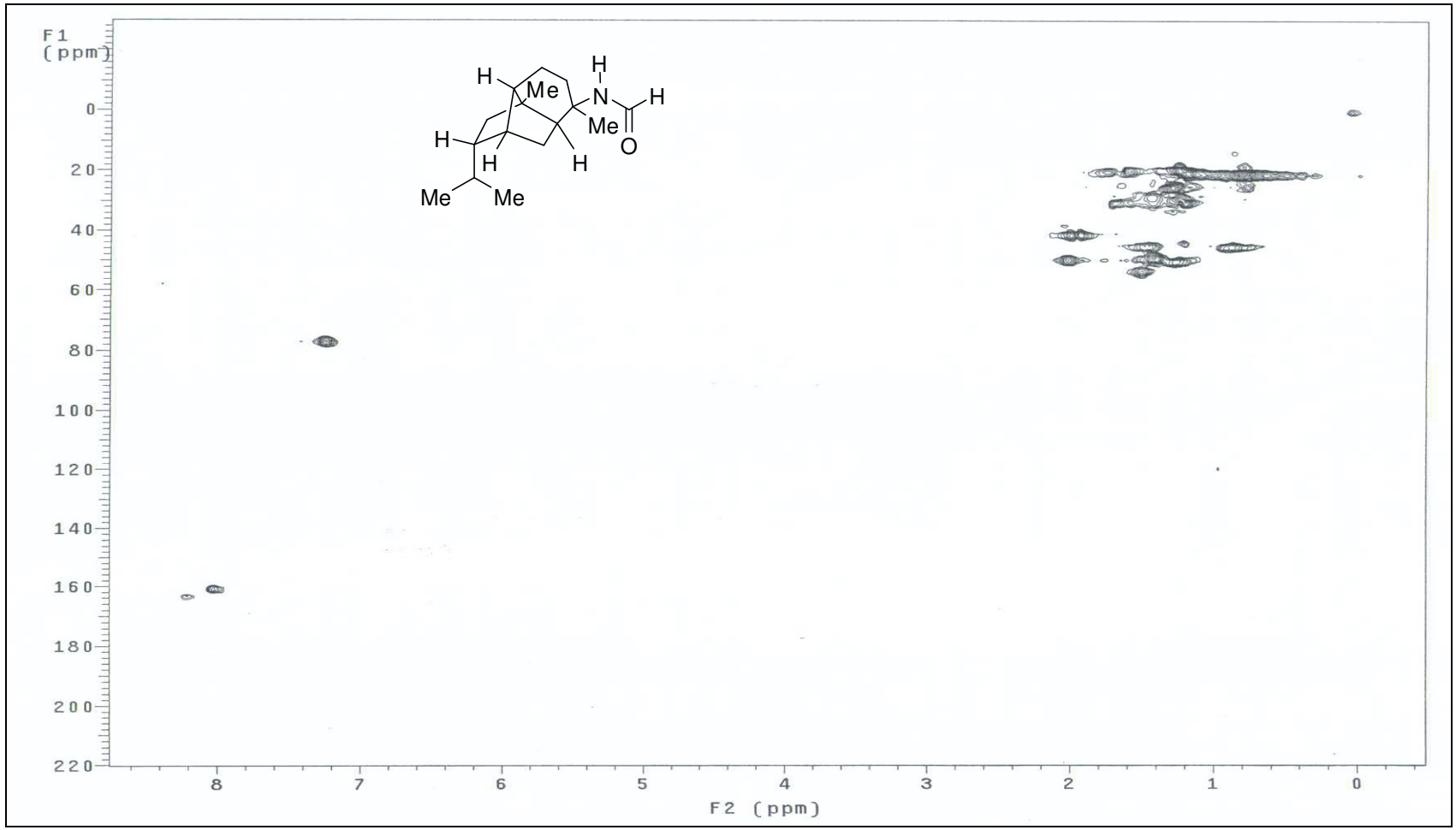
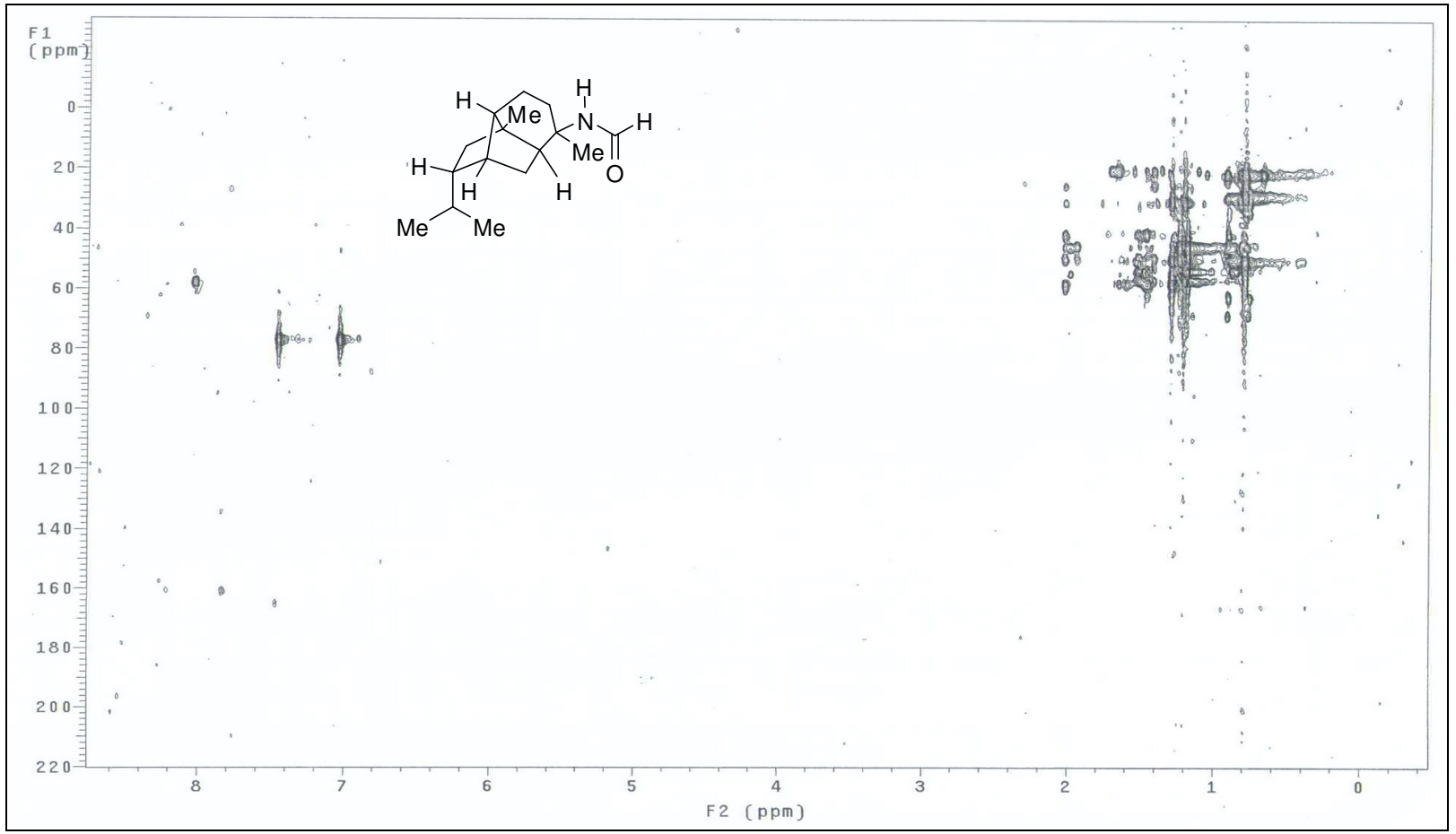


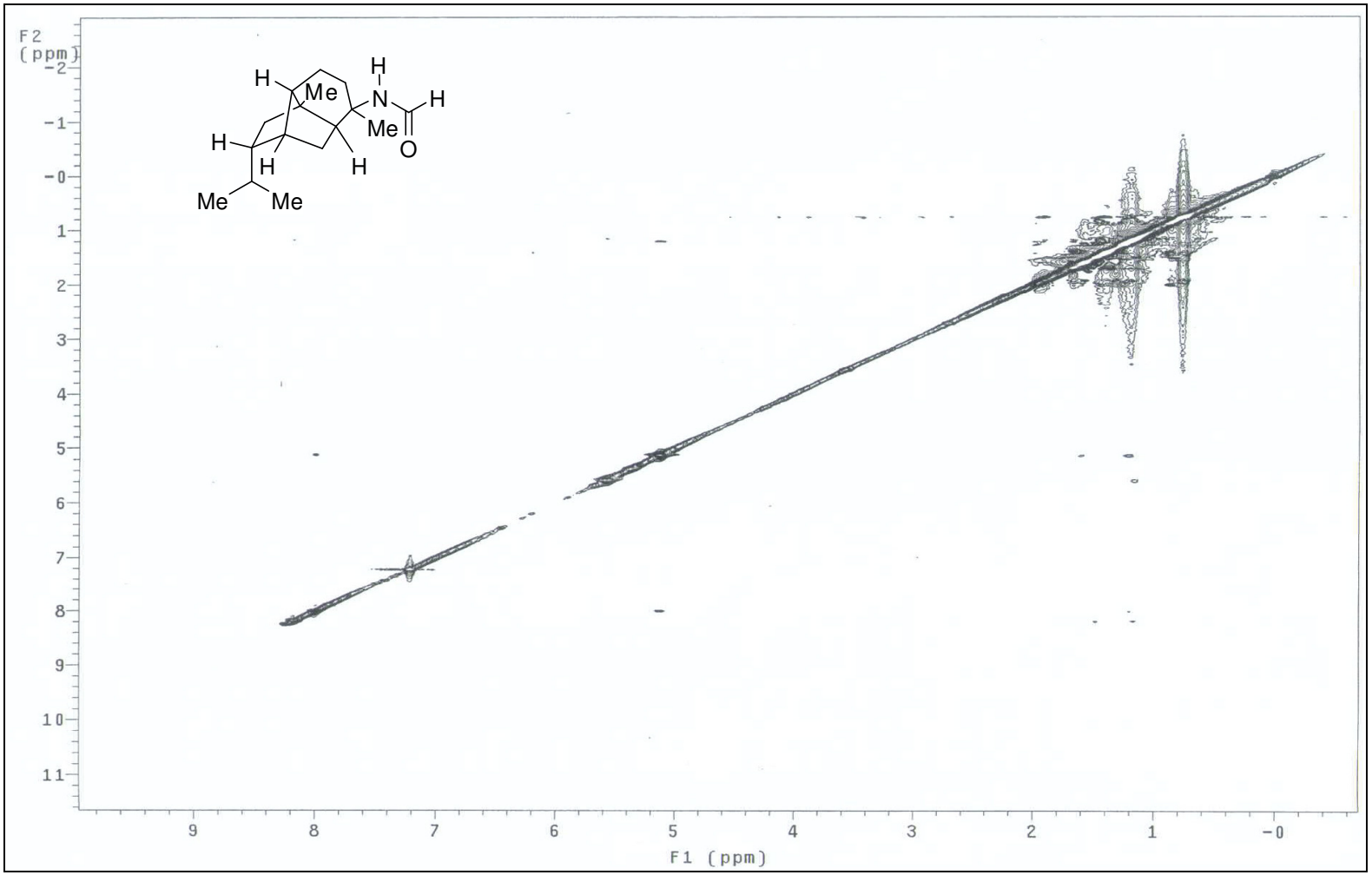
COSY ($CDCl_3$) spectrum of **1**



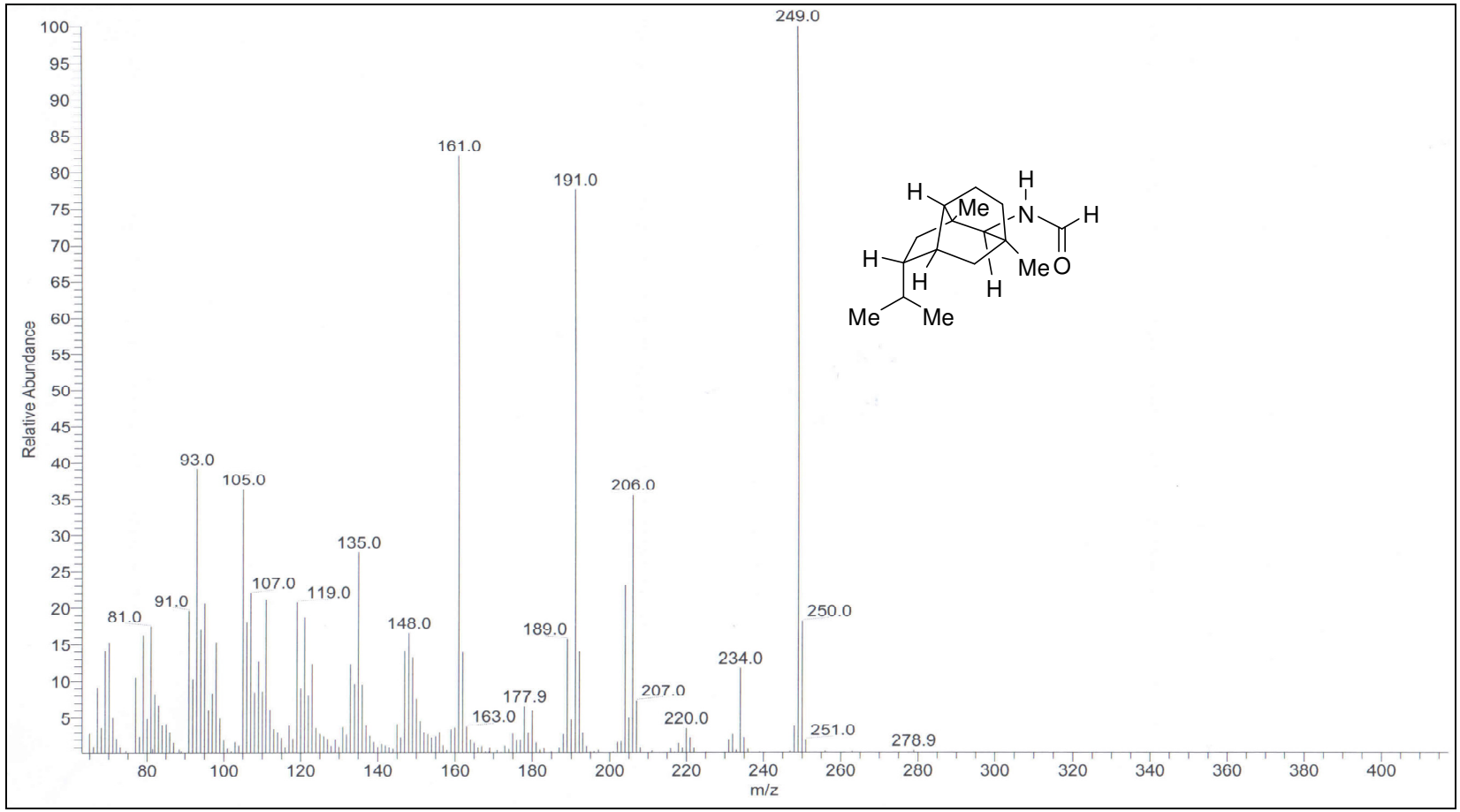
HMQC ($CDCl_3$) spectrum of **1**



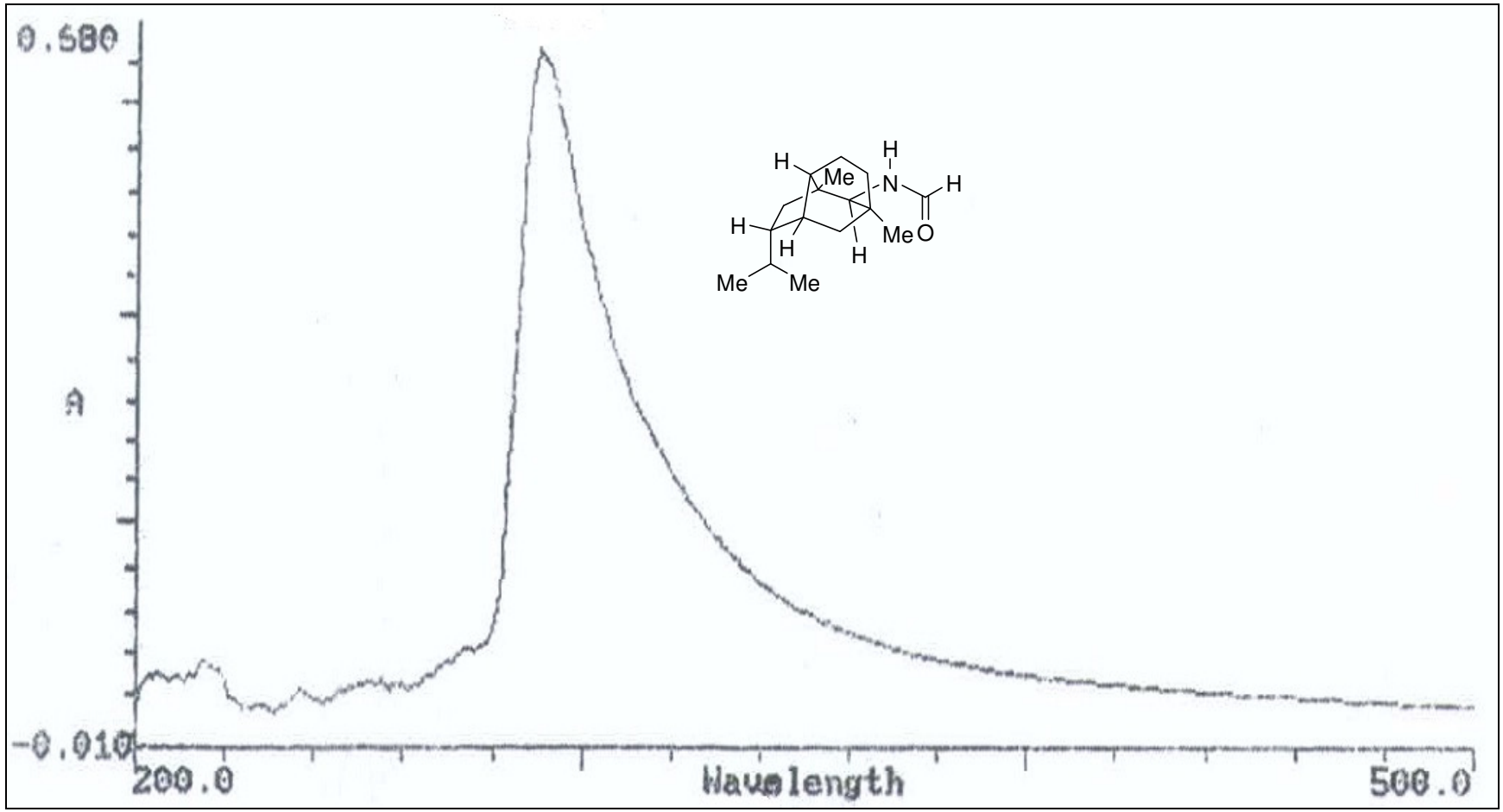
HMBC ($CDCl_3$) spectrum of **1**



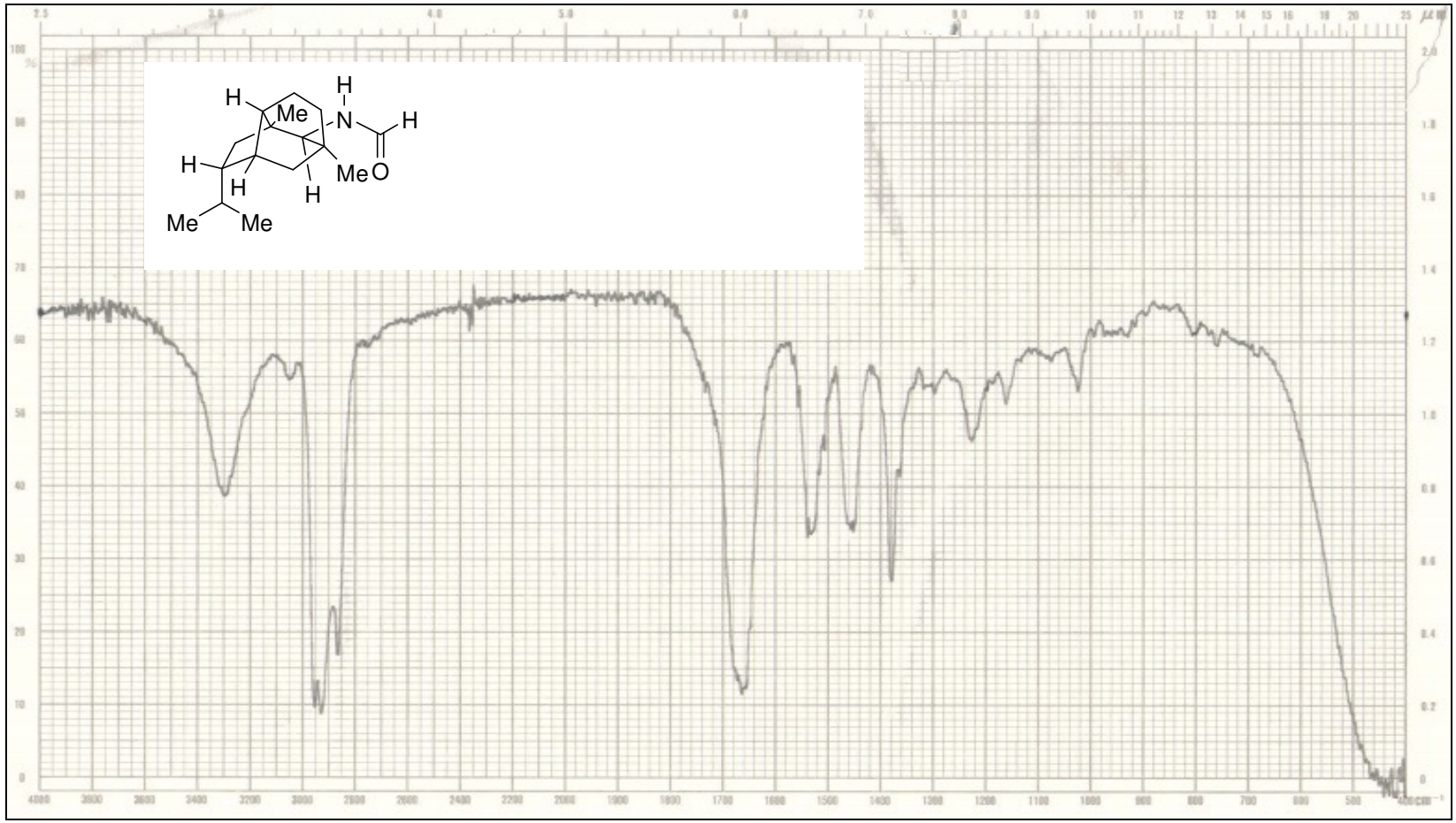
NOESY ($CDCl_3$) spectrum of **1**



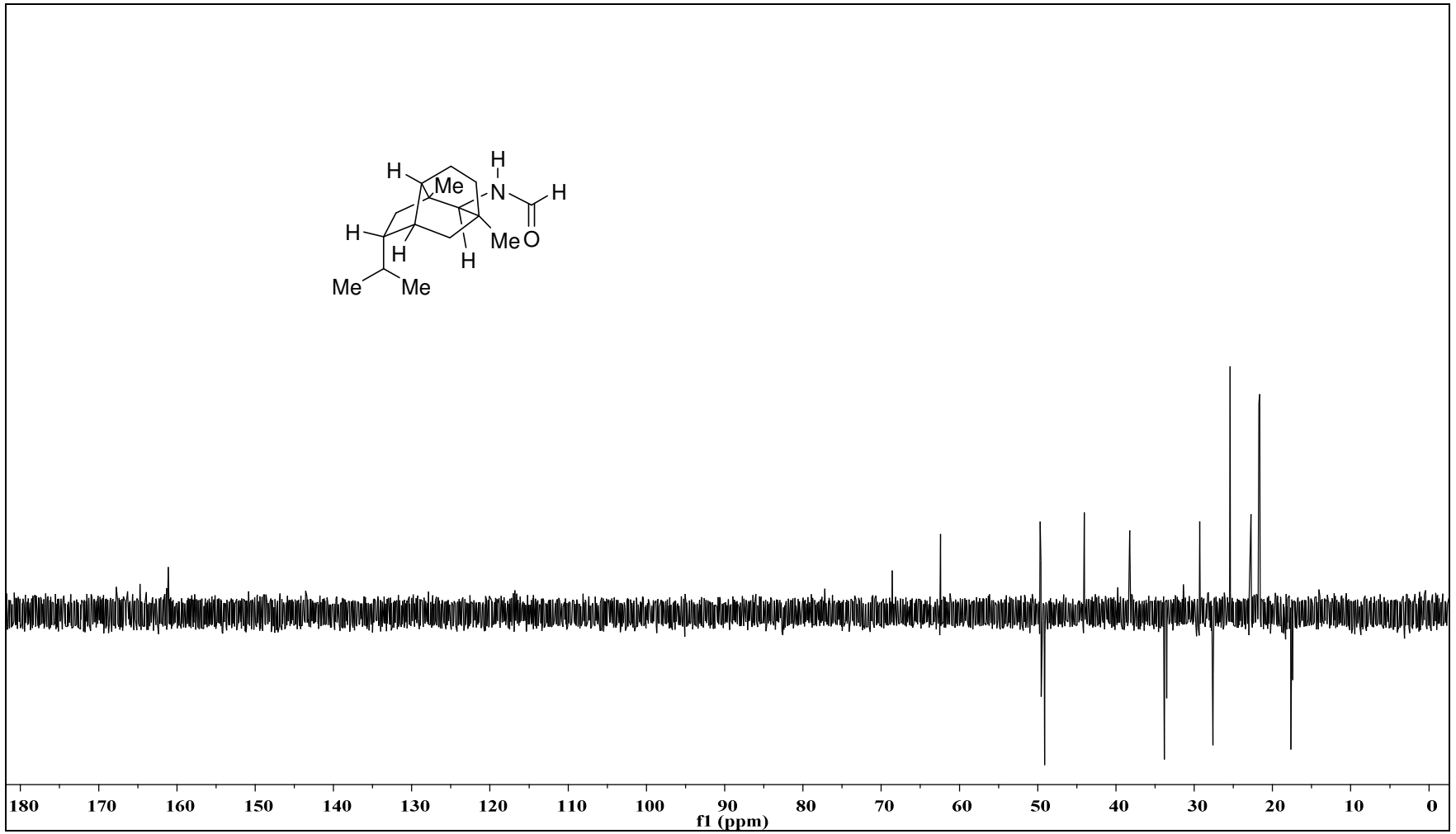
EIMS spectrum of **2**



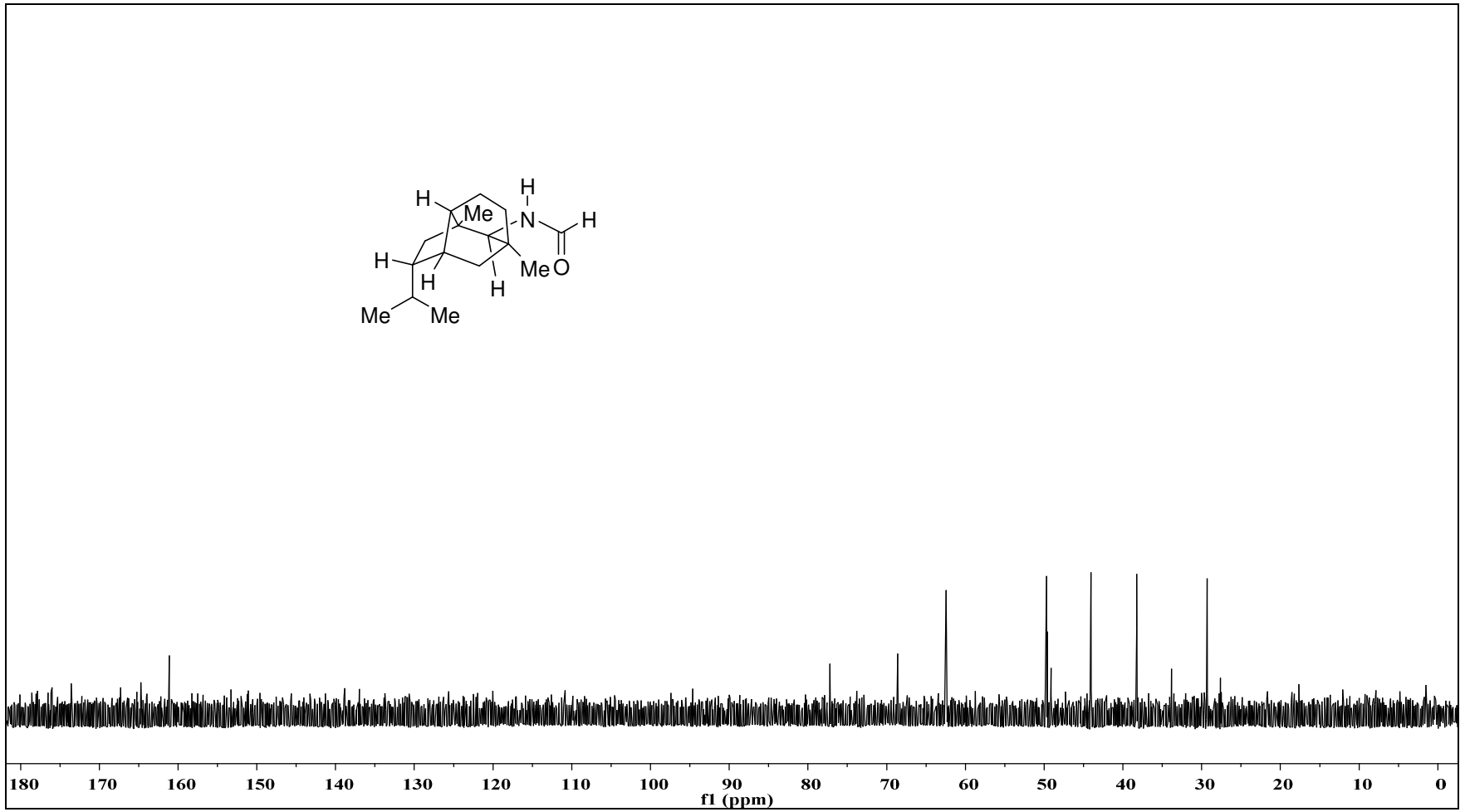
UV (MeOH) spectrum of **2**



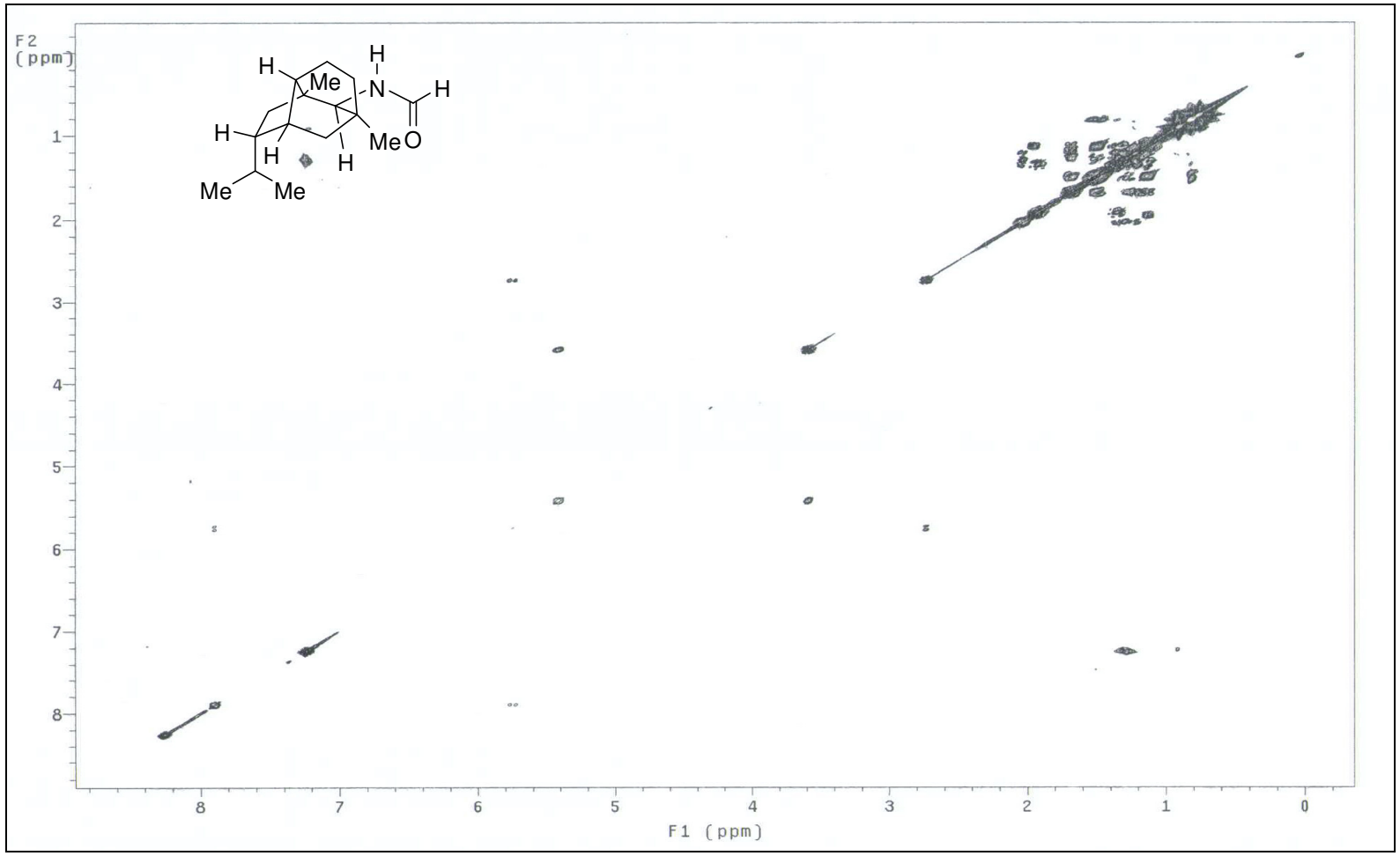
IR (neat) spectrum of **2**



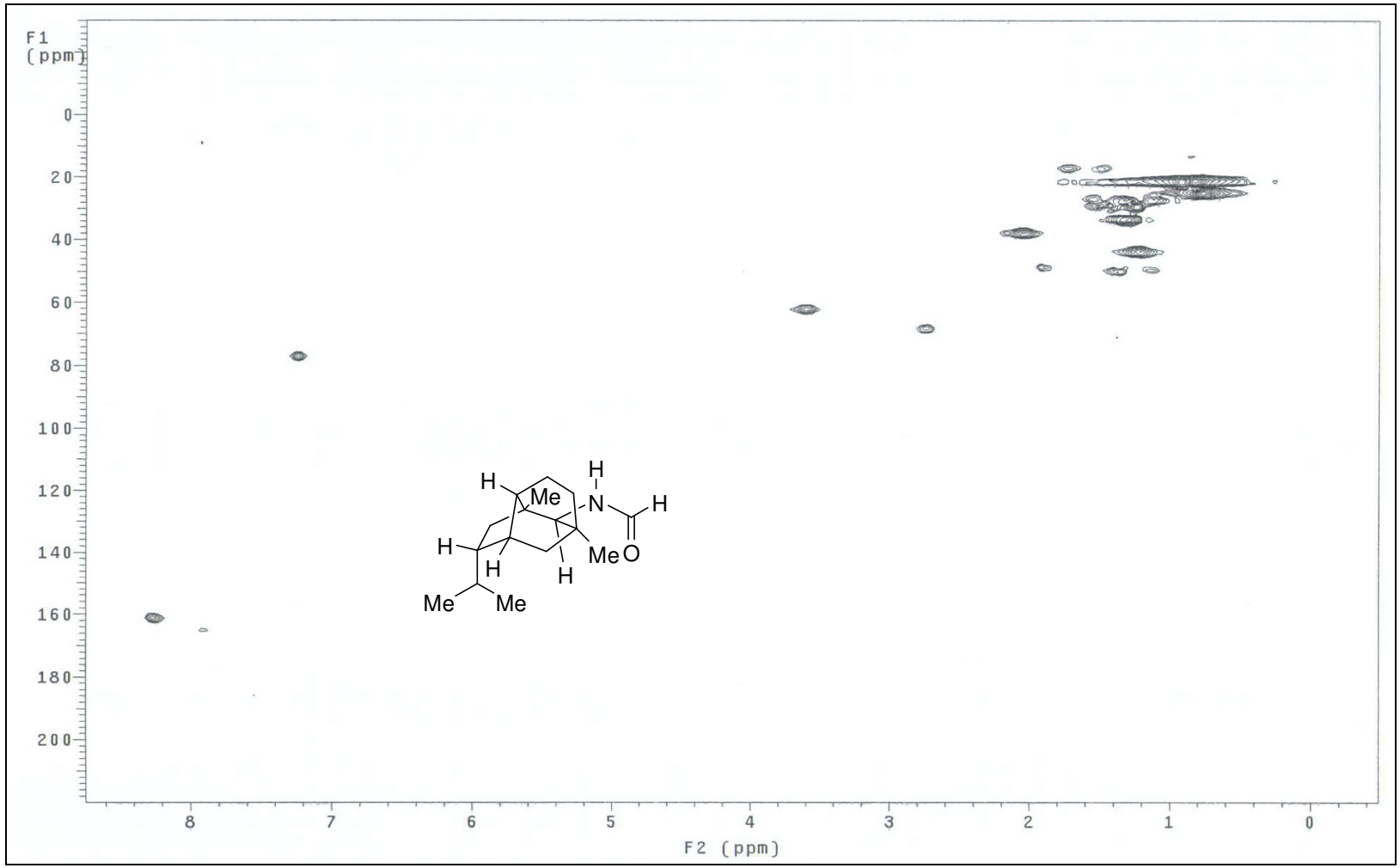
DEPT 135 ($CDCl_3$) spectrum of **2**



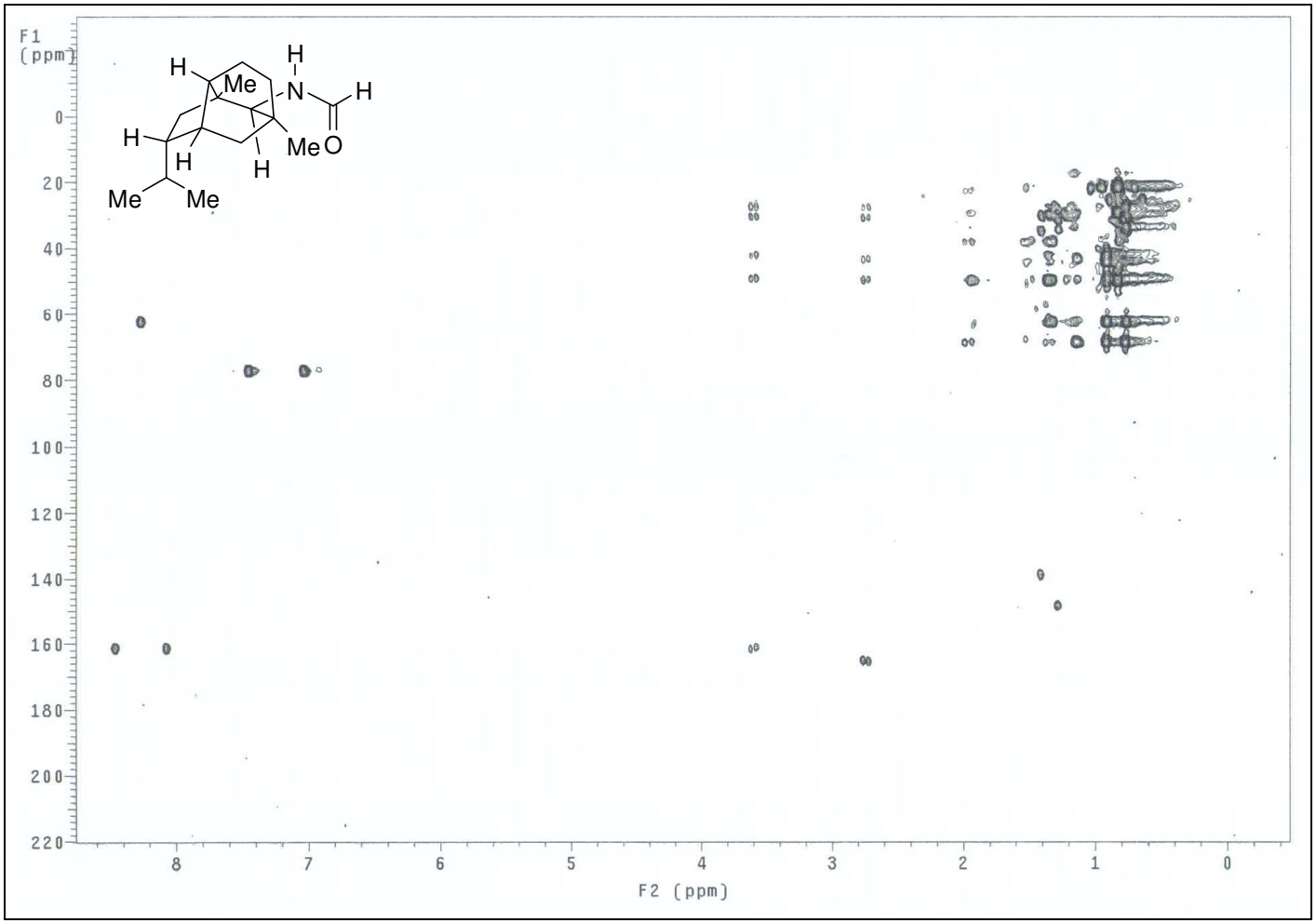
DEPT 90 ($CDCl_3$) spectrum of **2**



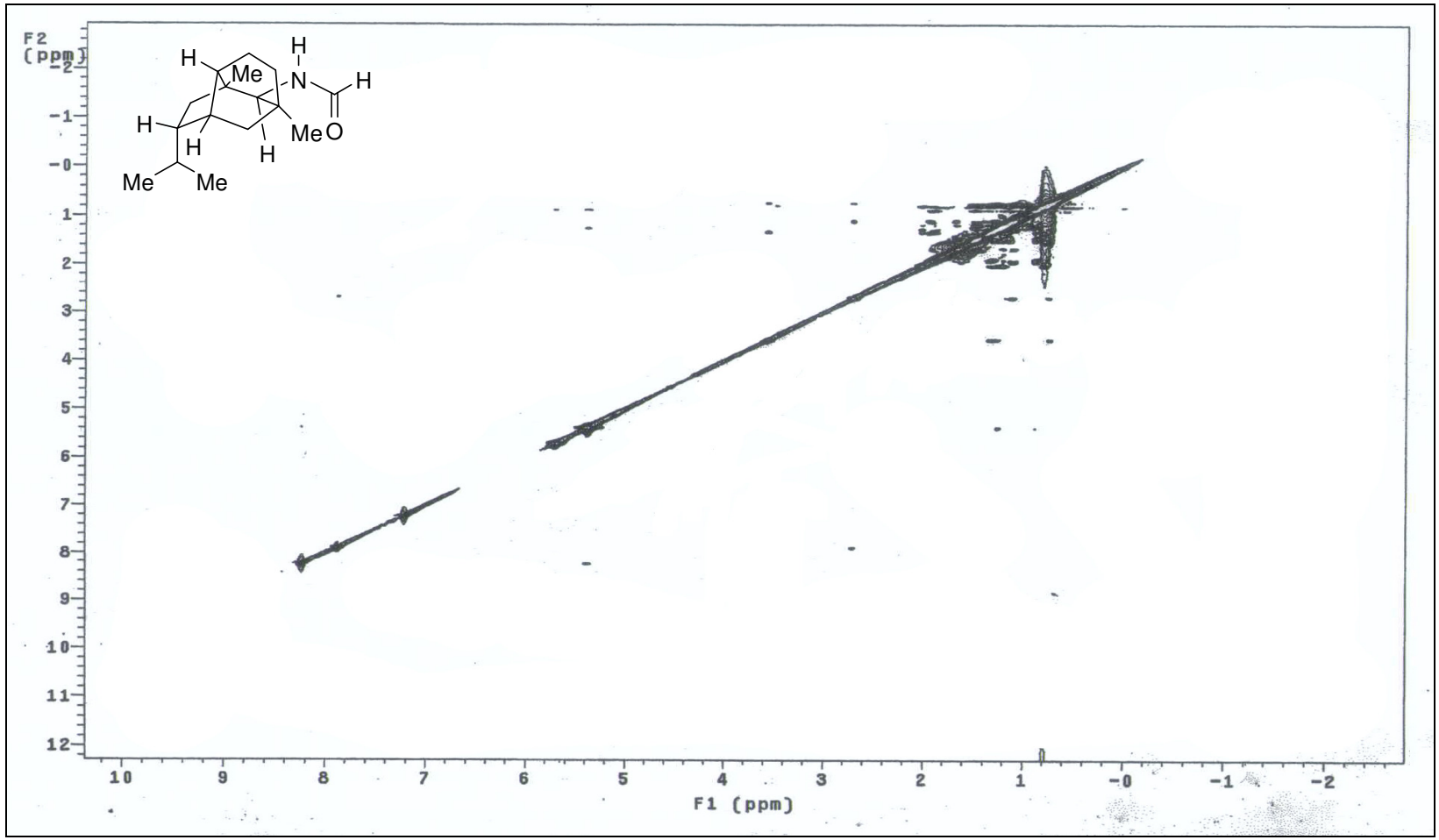
COSY ($CDCl_3$) spectrum of **2**



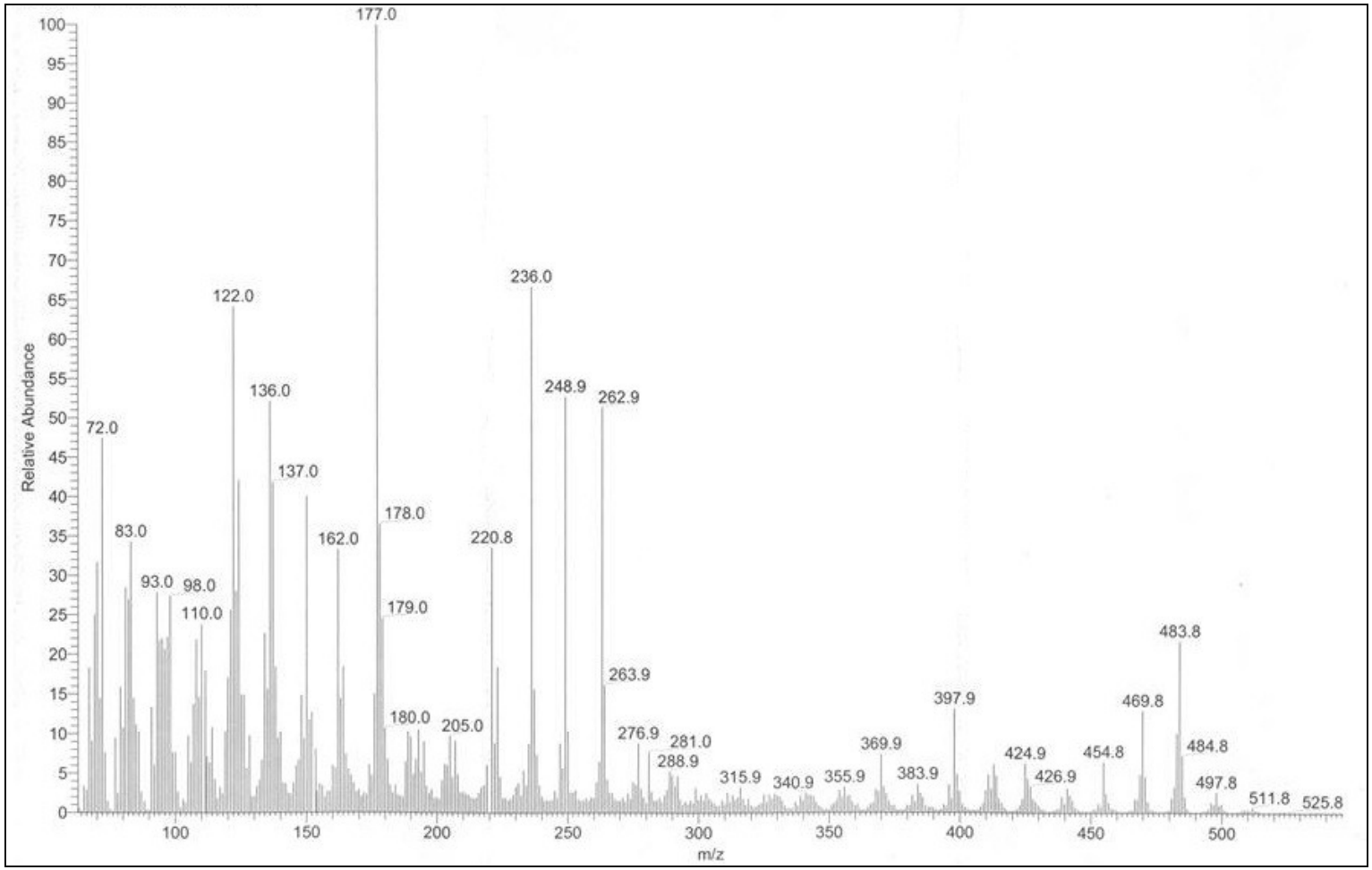
HMQC ($CDCl_3$) spectrum of **2**



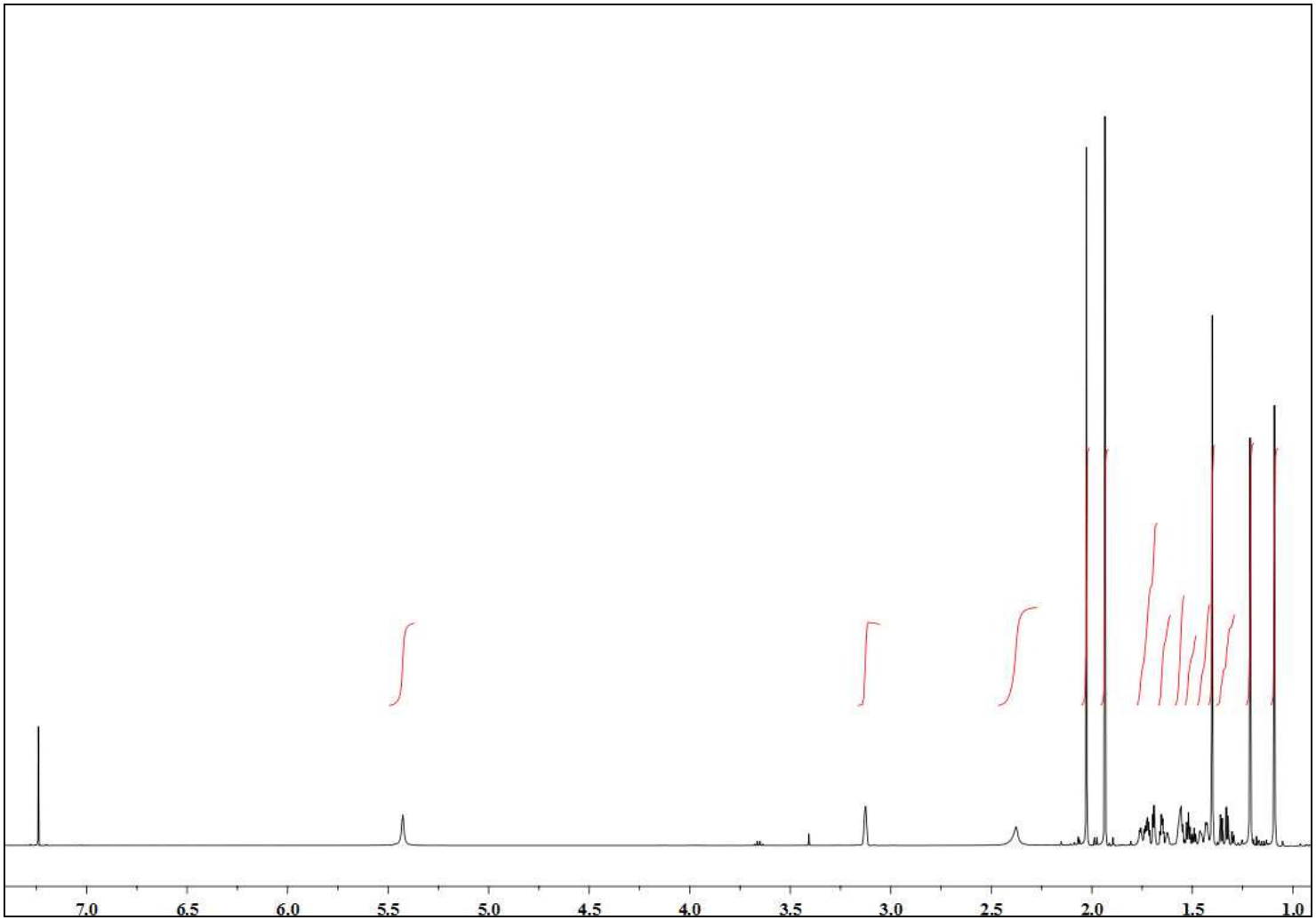
HMBC ($CDCl_3$) spectrum of **2**



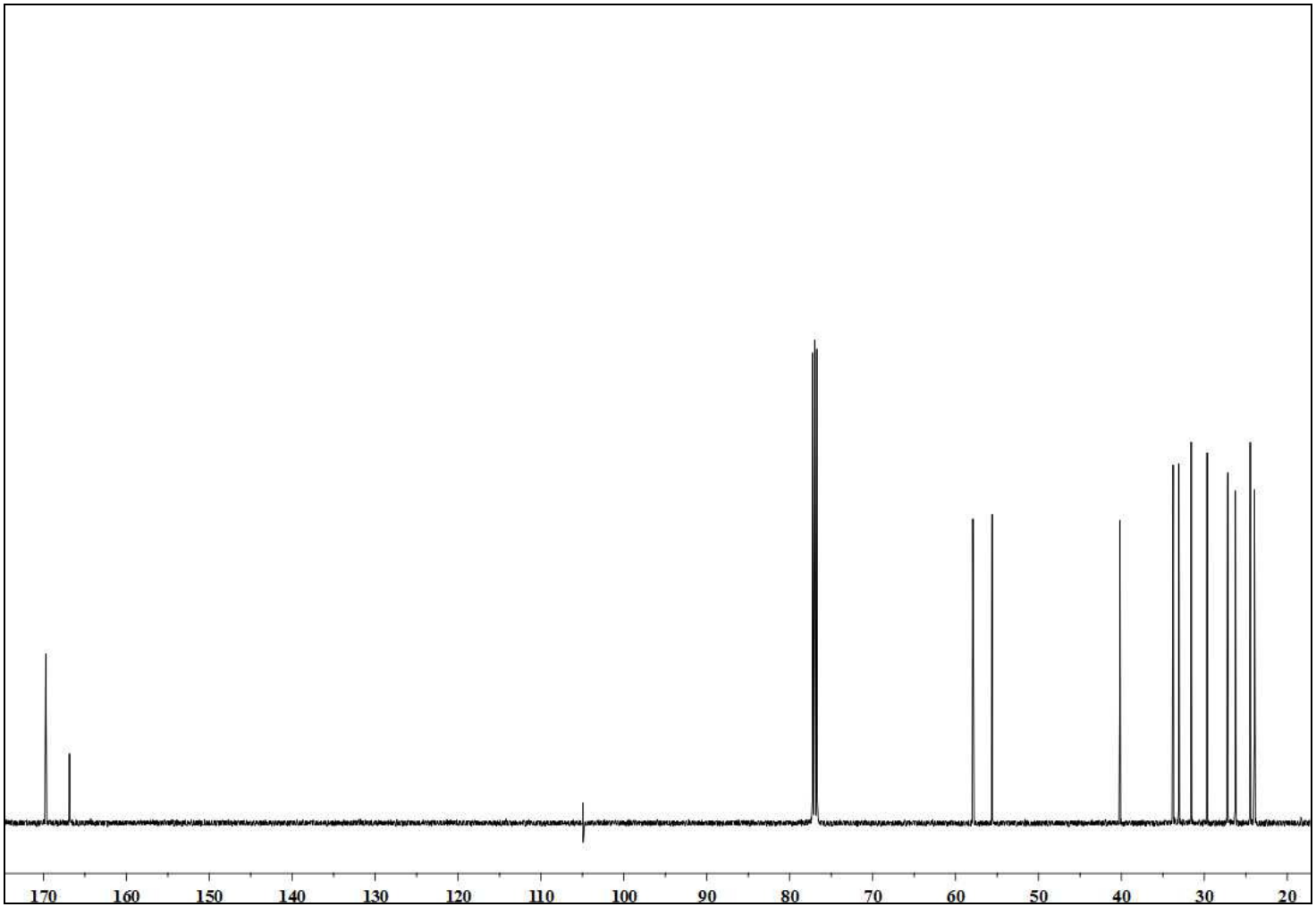
NOESY ($CDCl_3$) spectrum of **2**



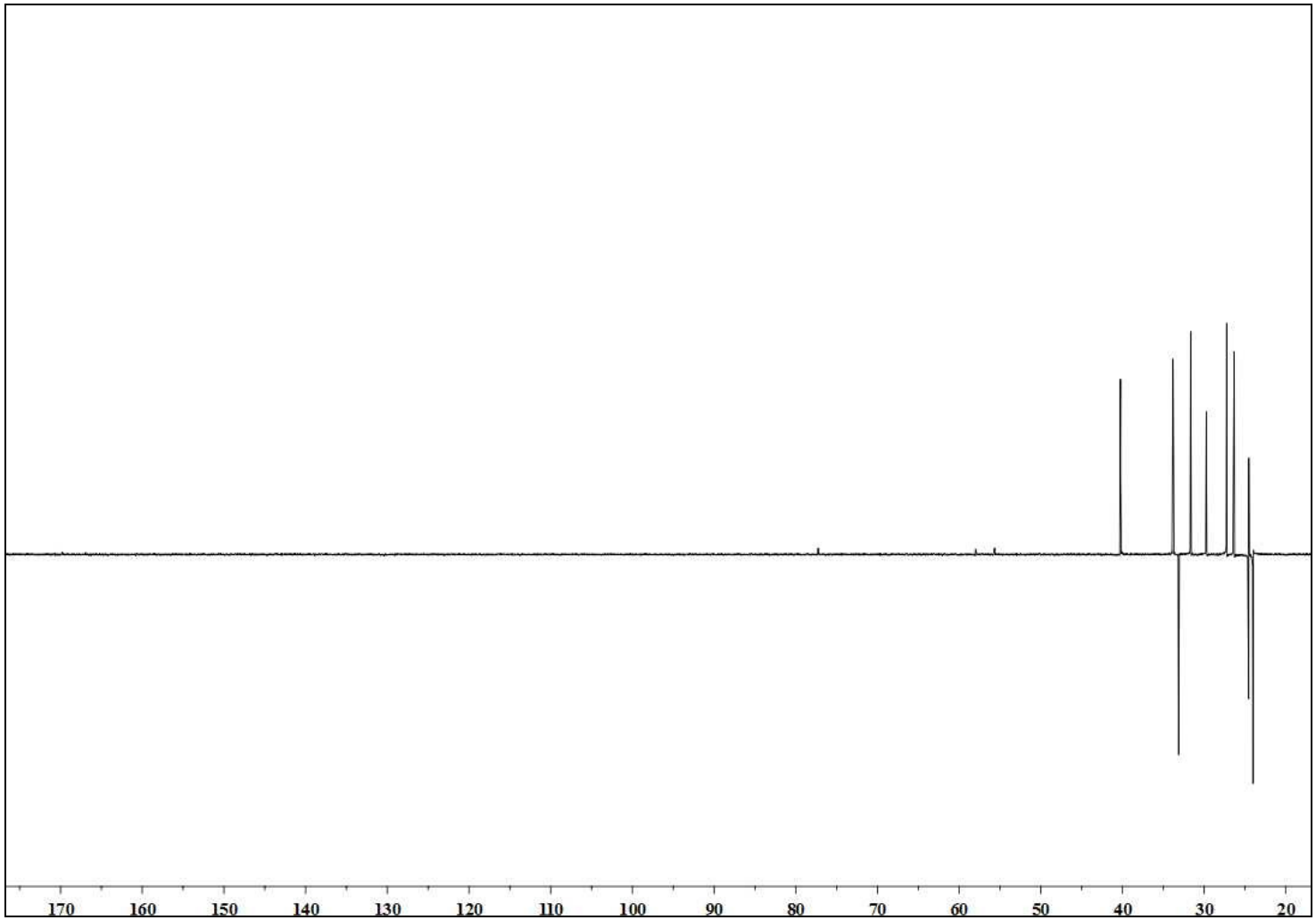
EIMS spectrum of **3**



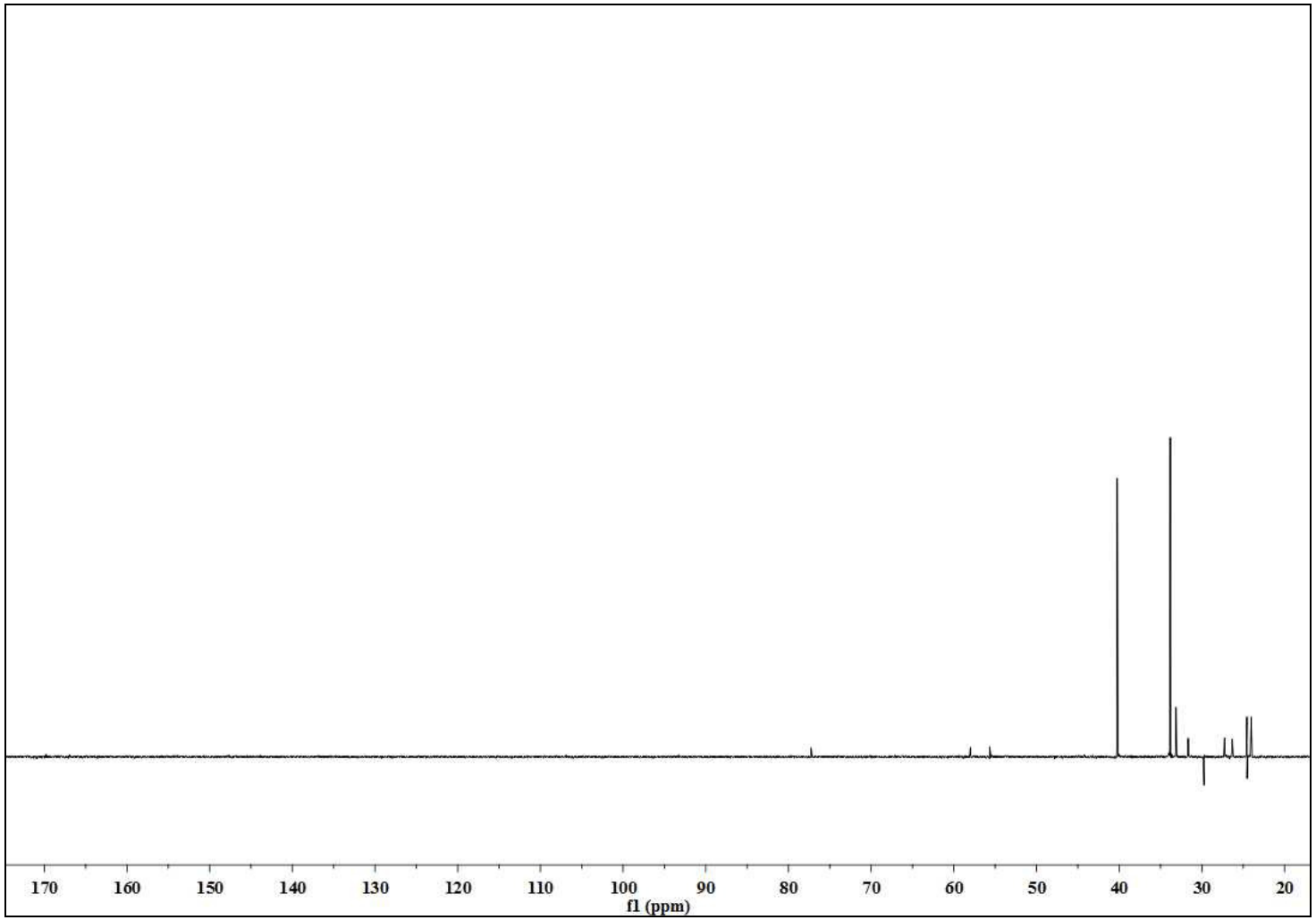
^{1}H-NMR spectrum of **3** (500 MHz, $CDCl_3$)



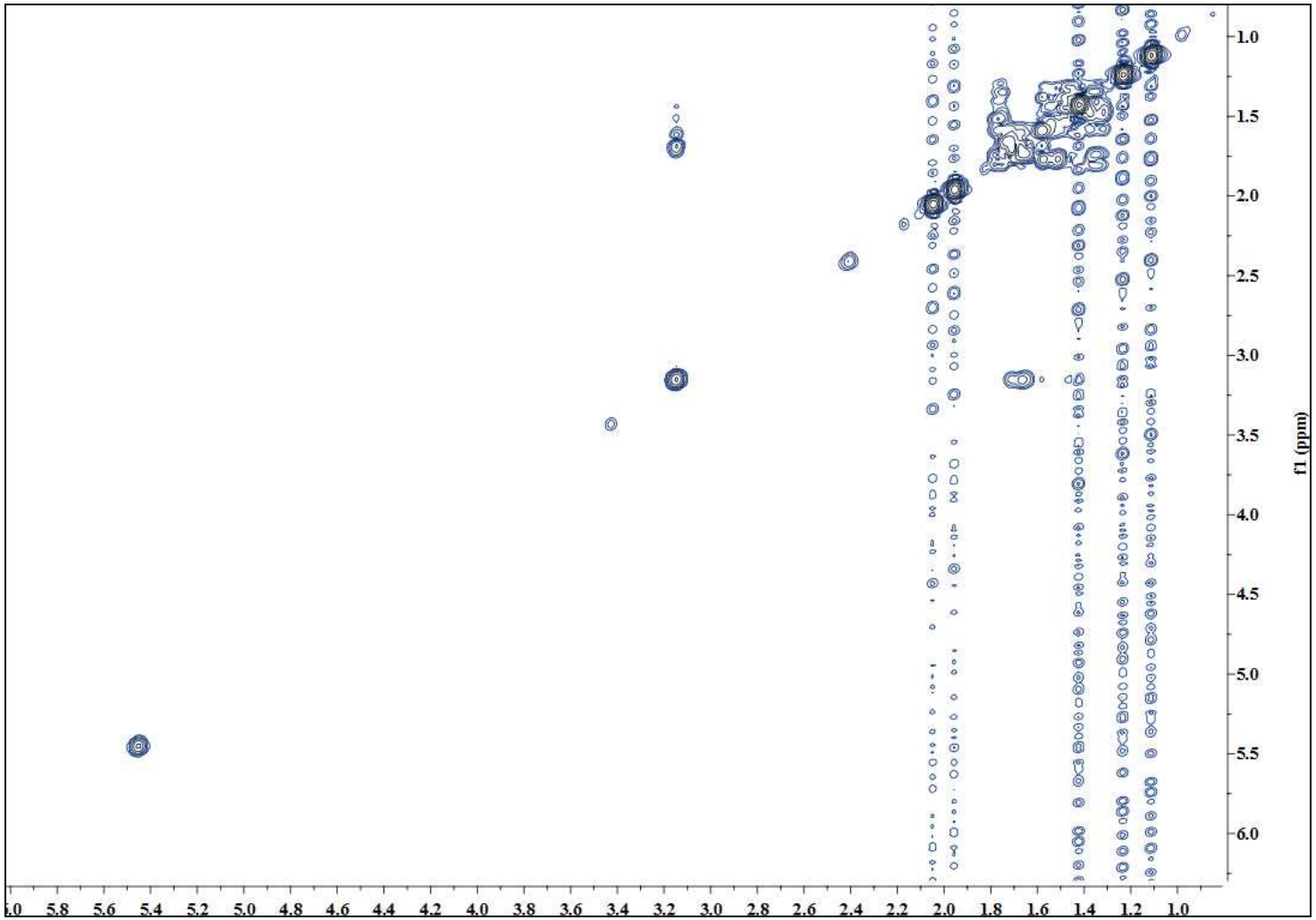
^{13}C-NMR spectrum of **3** (125 MHz, $CDCl_3$)



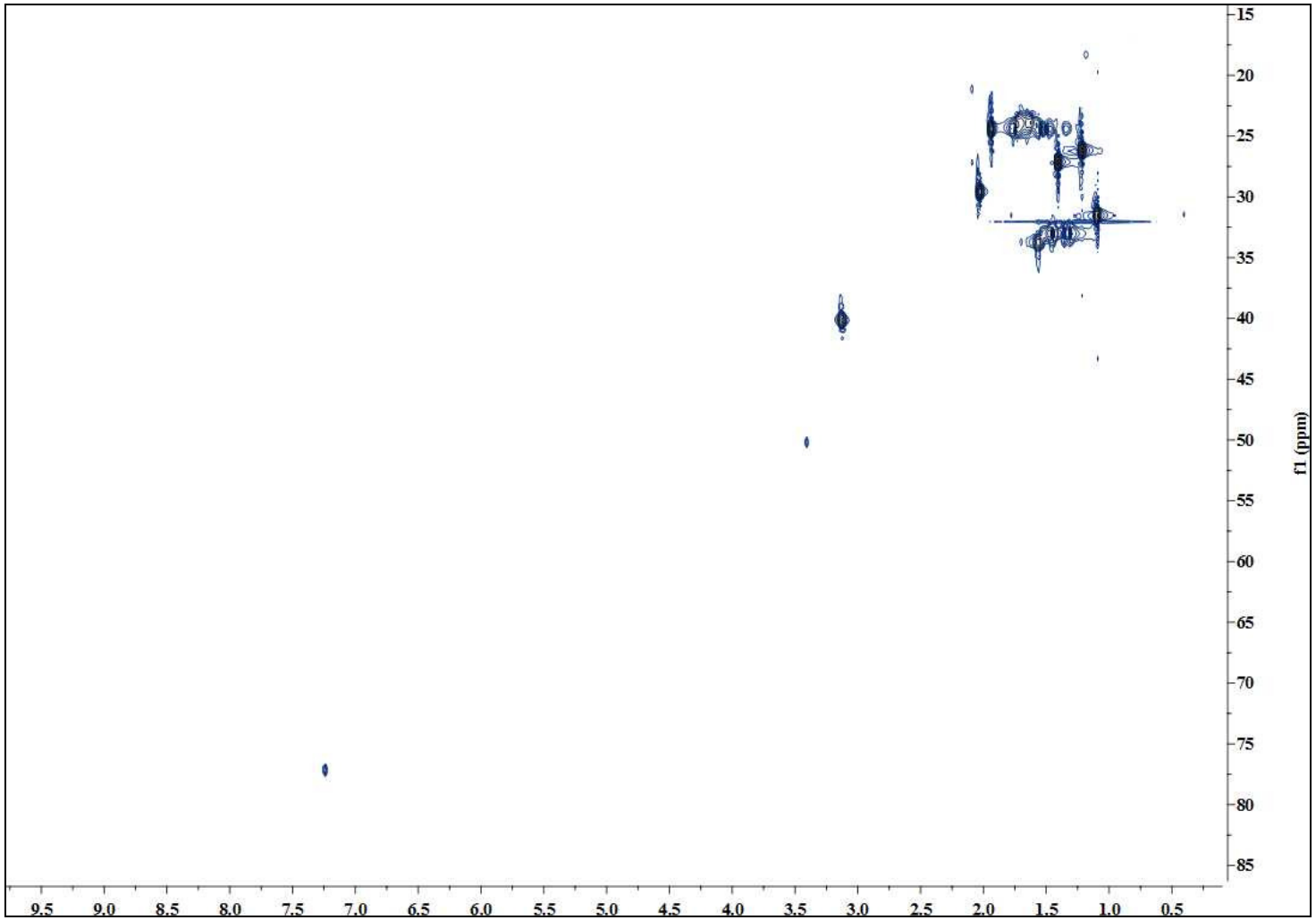
DEPT 135 ($CDCl_3$) spectrum of **3**



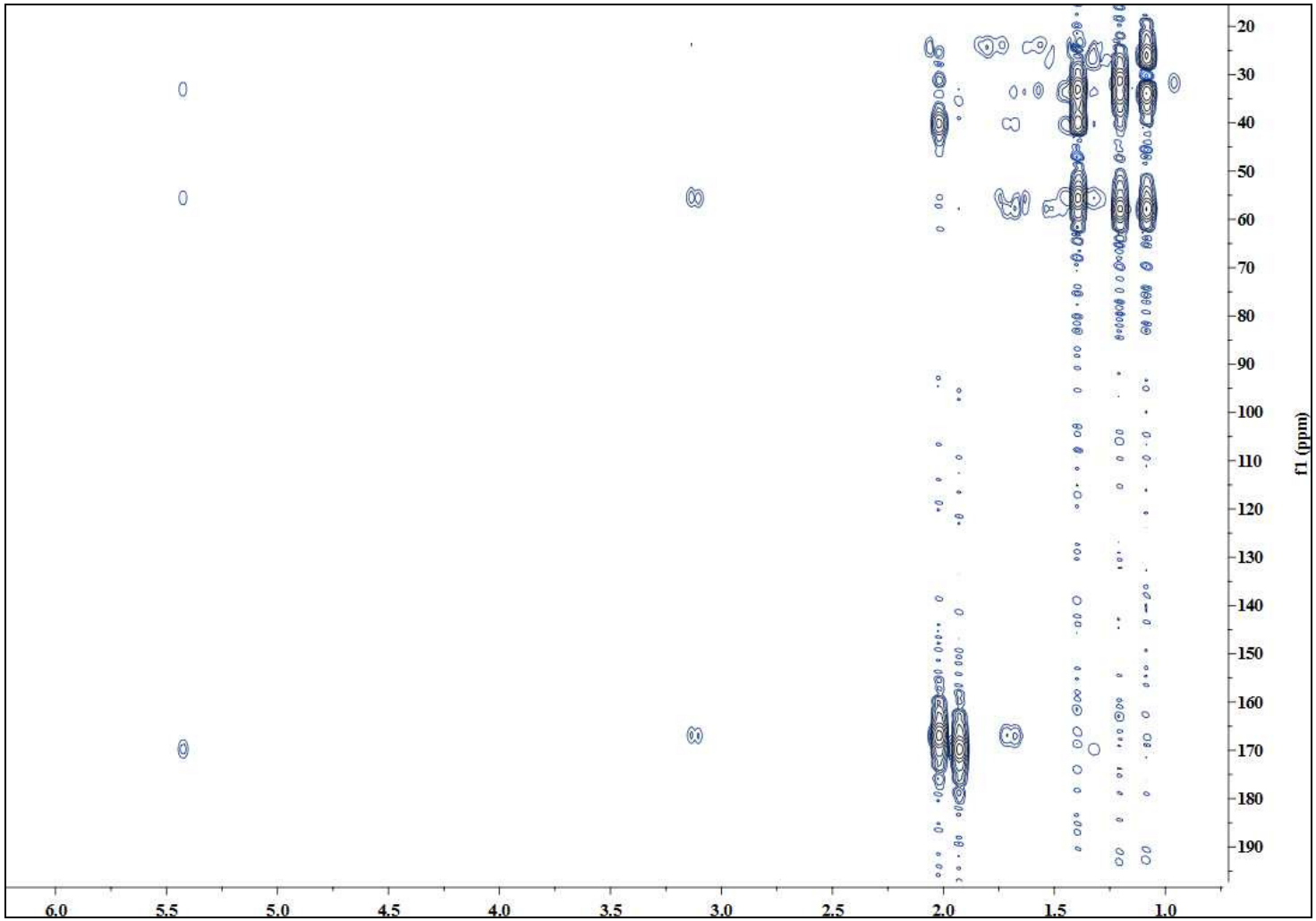
DEPT 90 ($CDCl_3$) spectrum of **3**



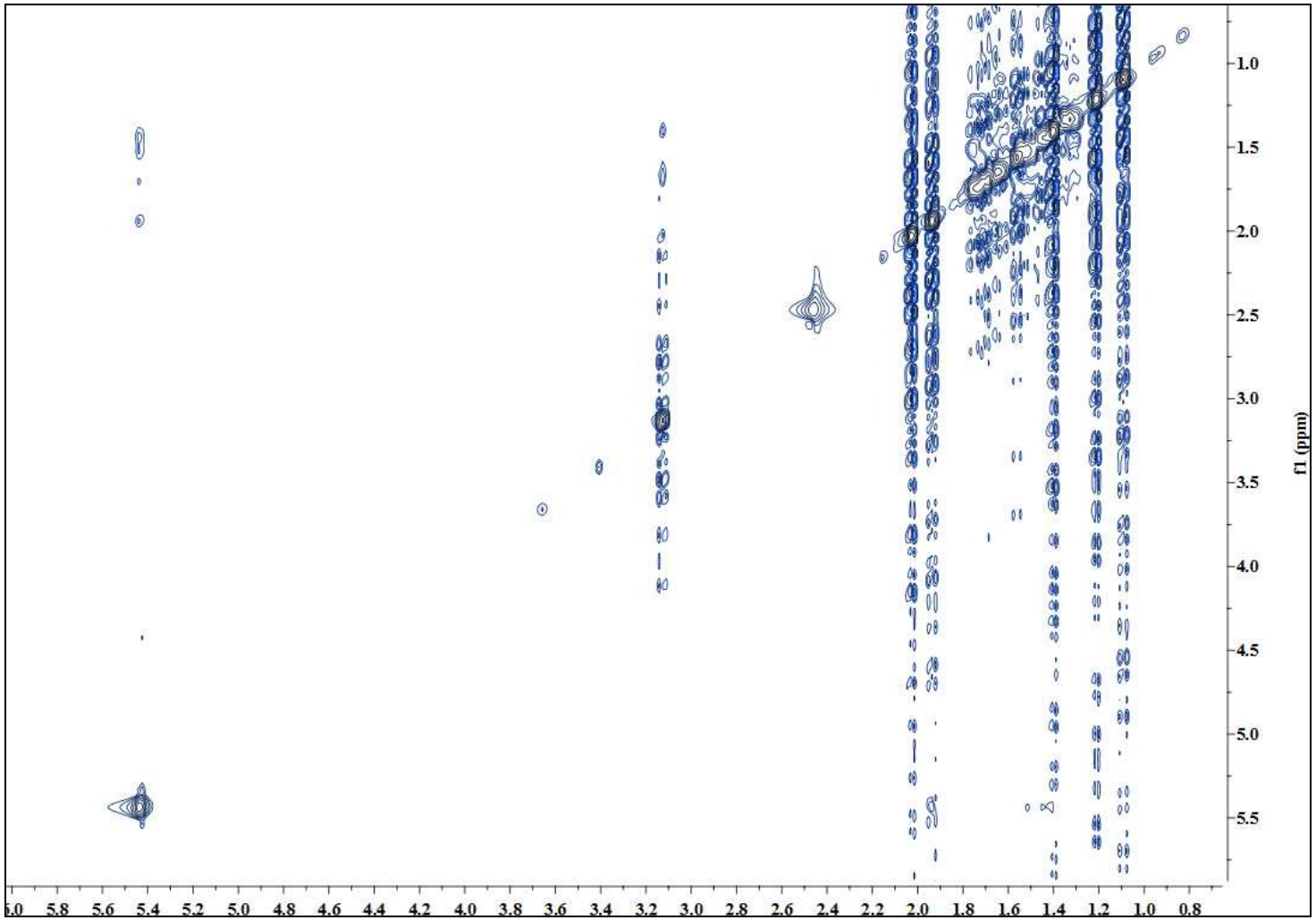
COSY ($CDCl_3$) spectrum of **3**



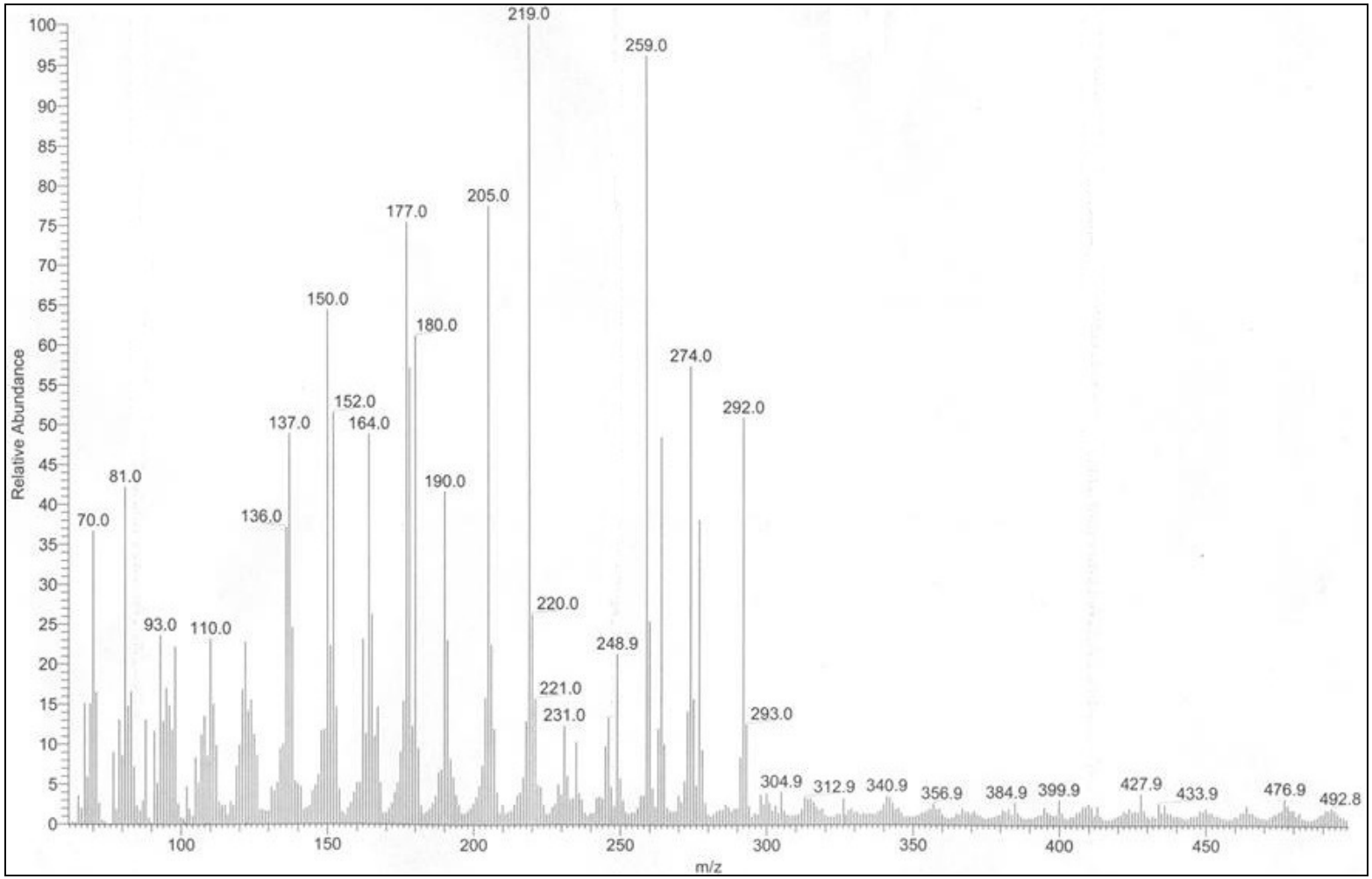
HMQC ($CDCl_3$) spectrum of 3



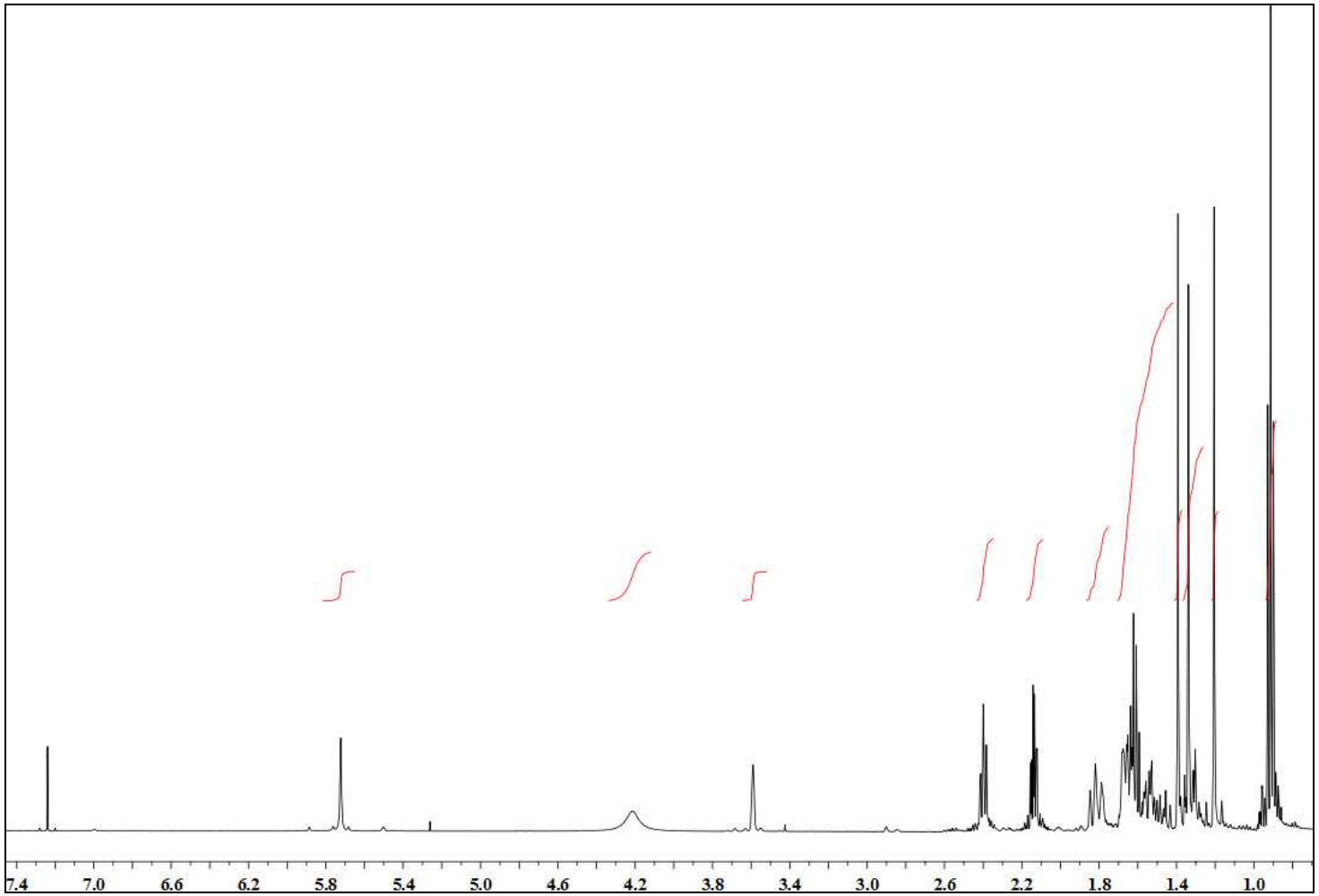
HMBC ($CDCl_3$) spectrum of **3**



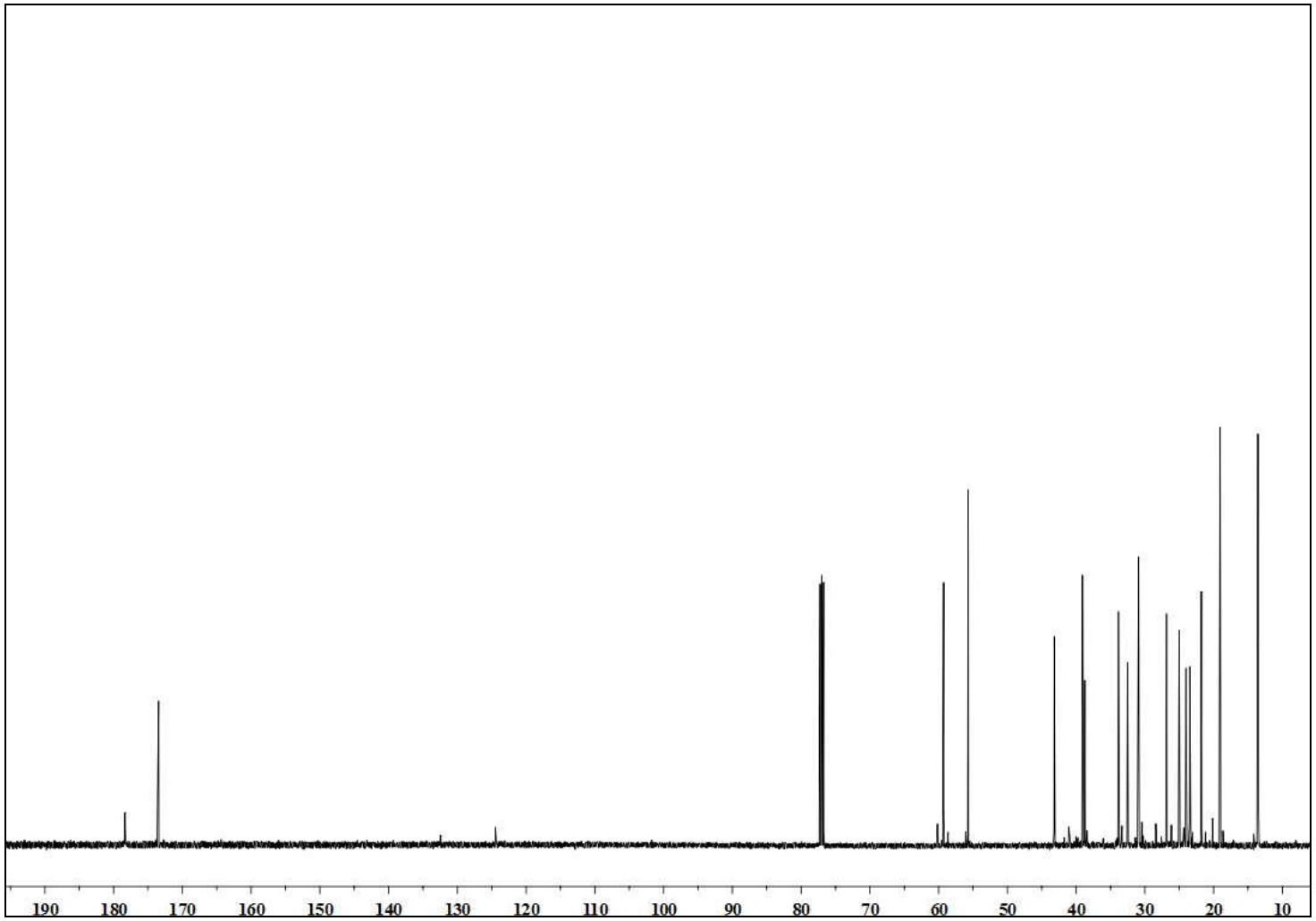
NOESY ($CDCl_3$) spectrum of **3**



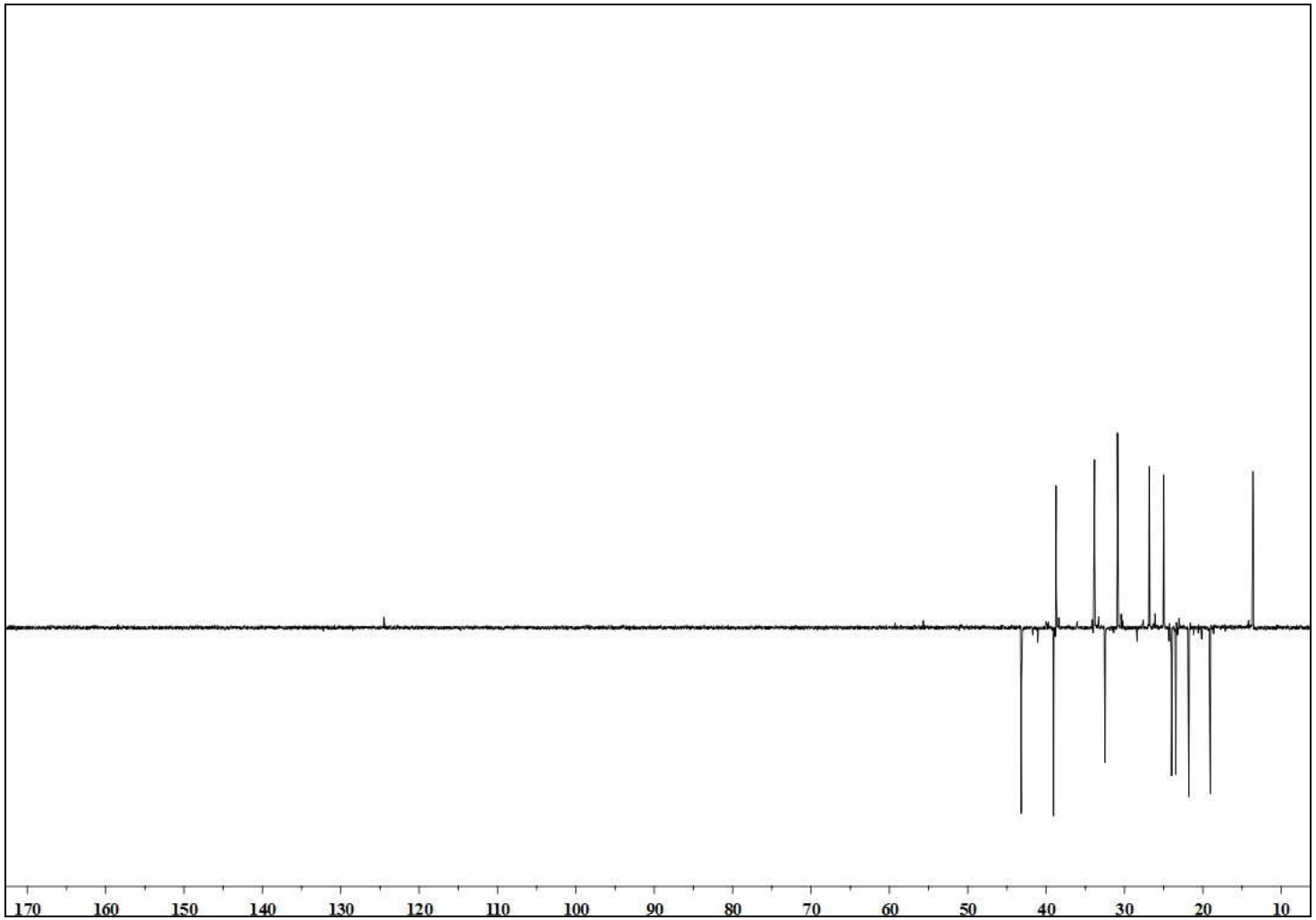
EIMS spectrum of **4**



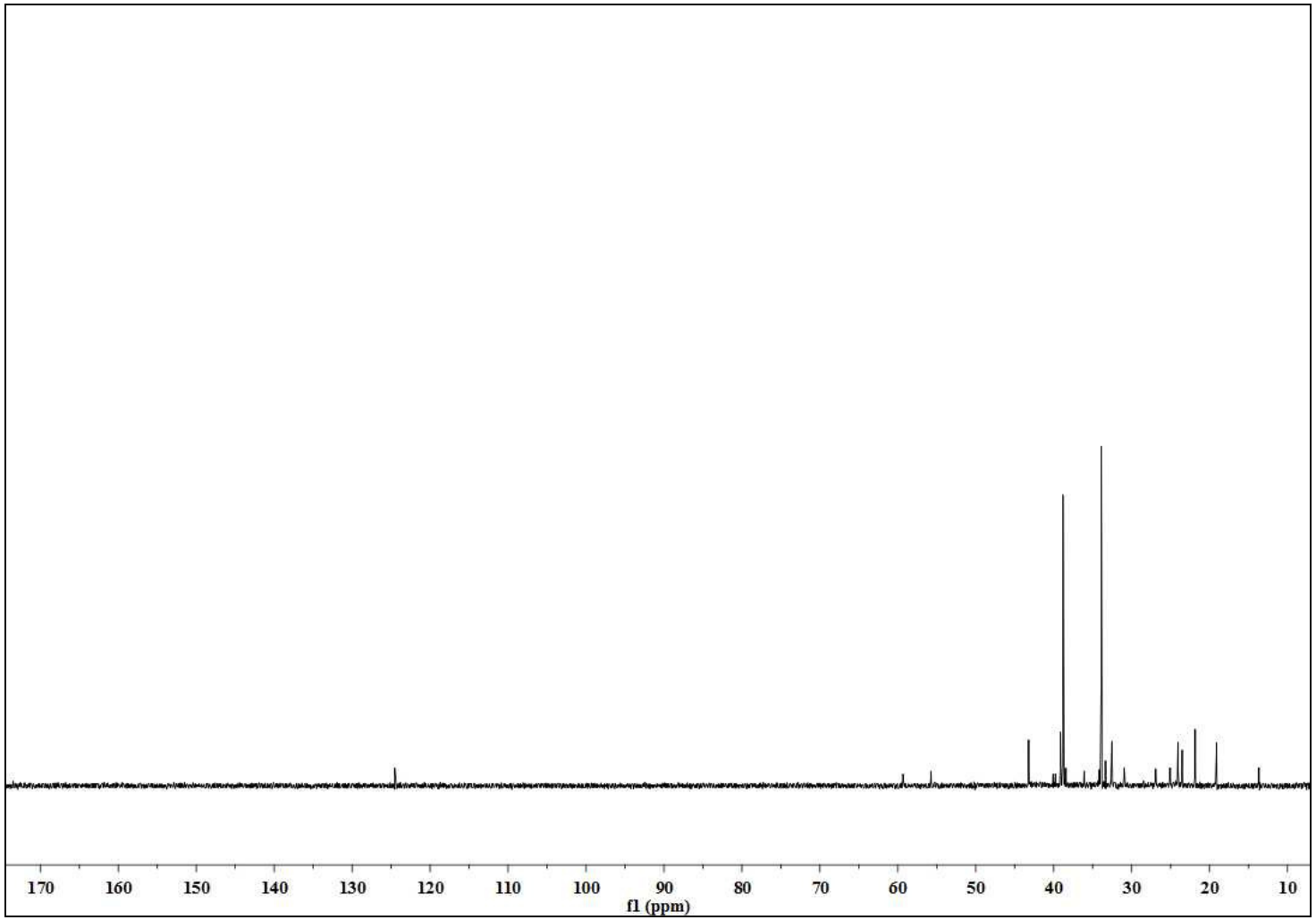
^{1}H-NMR spectrum of **4** (500 MHz, $CDCl_3$)



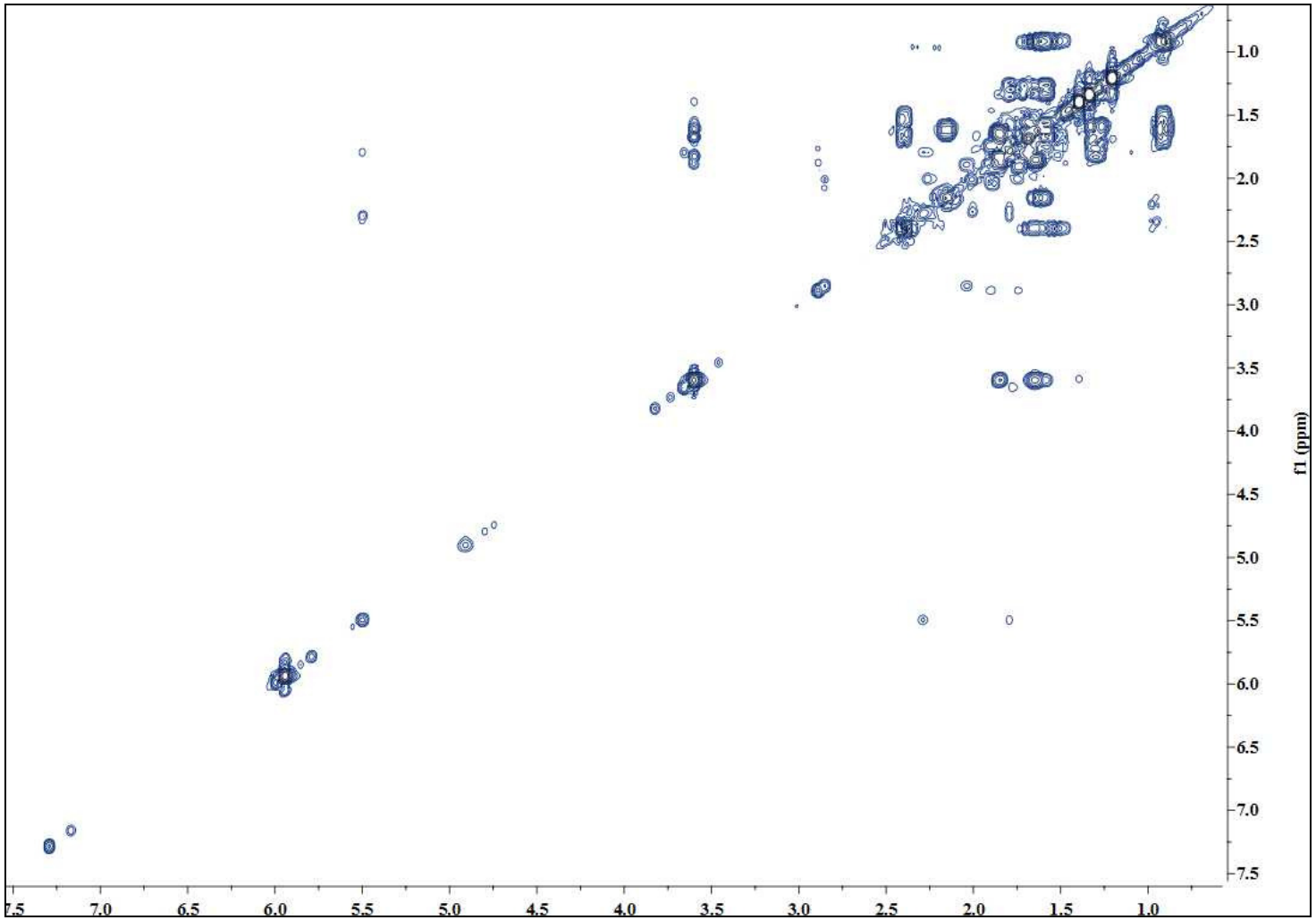
^{13}C-NMR spectrum of **4** (125 MHz, $CDCl_3$)



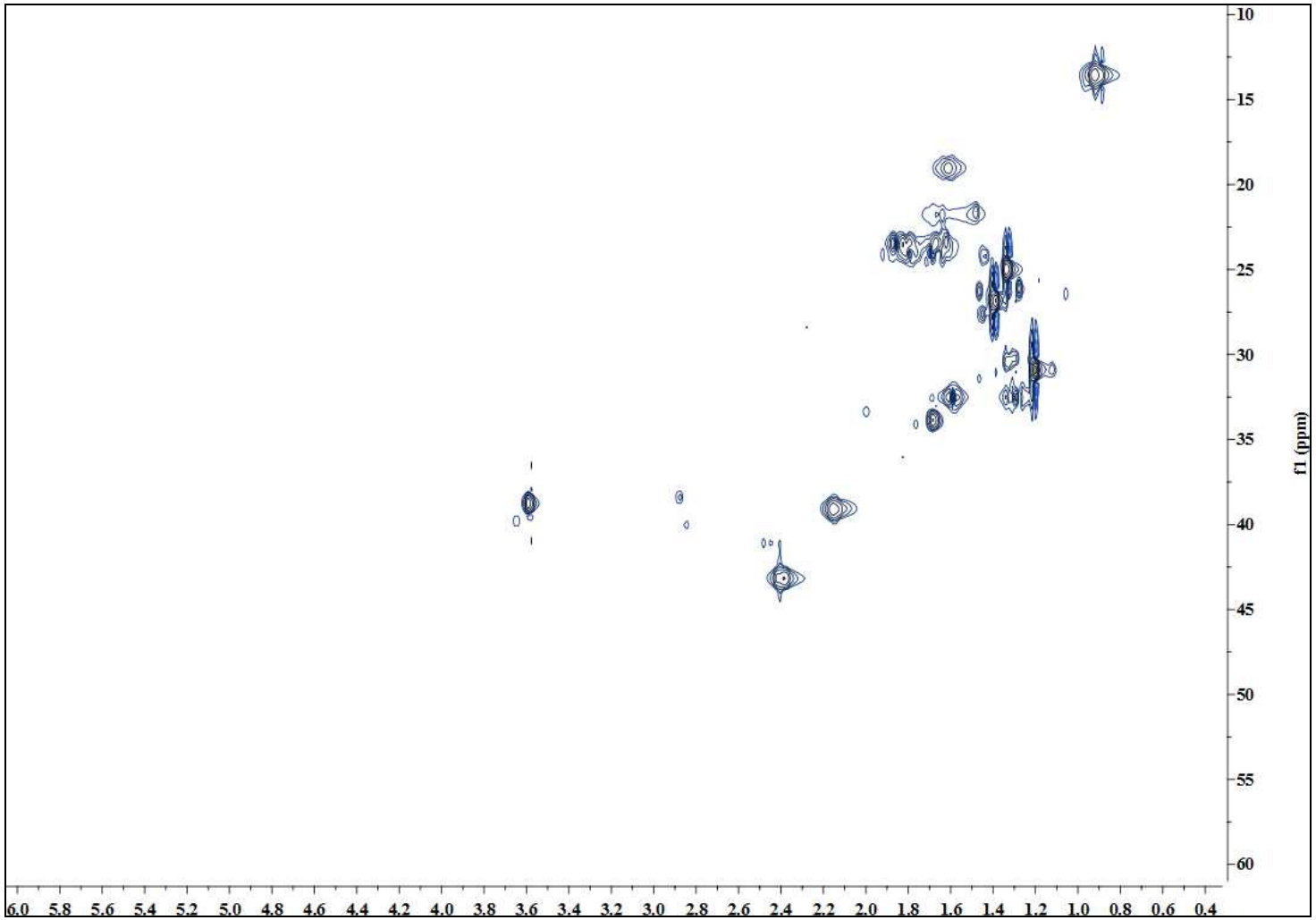
DEPT 135 ($CDCl_3$) spectrum of **4**



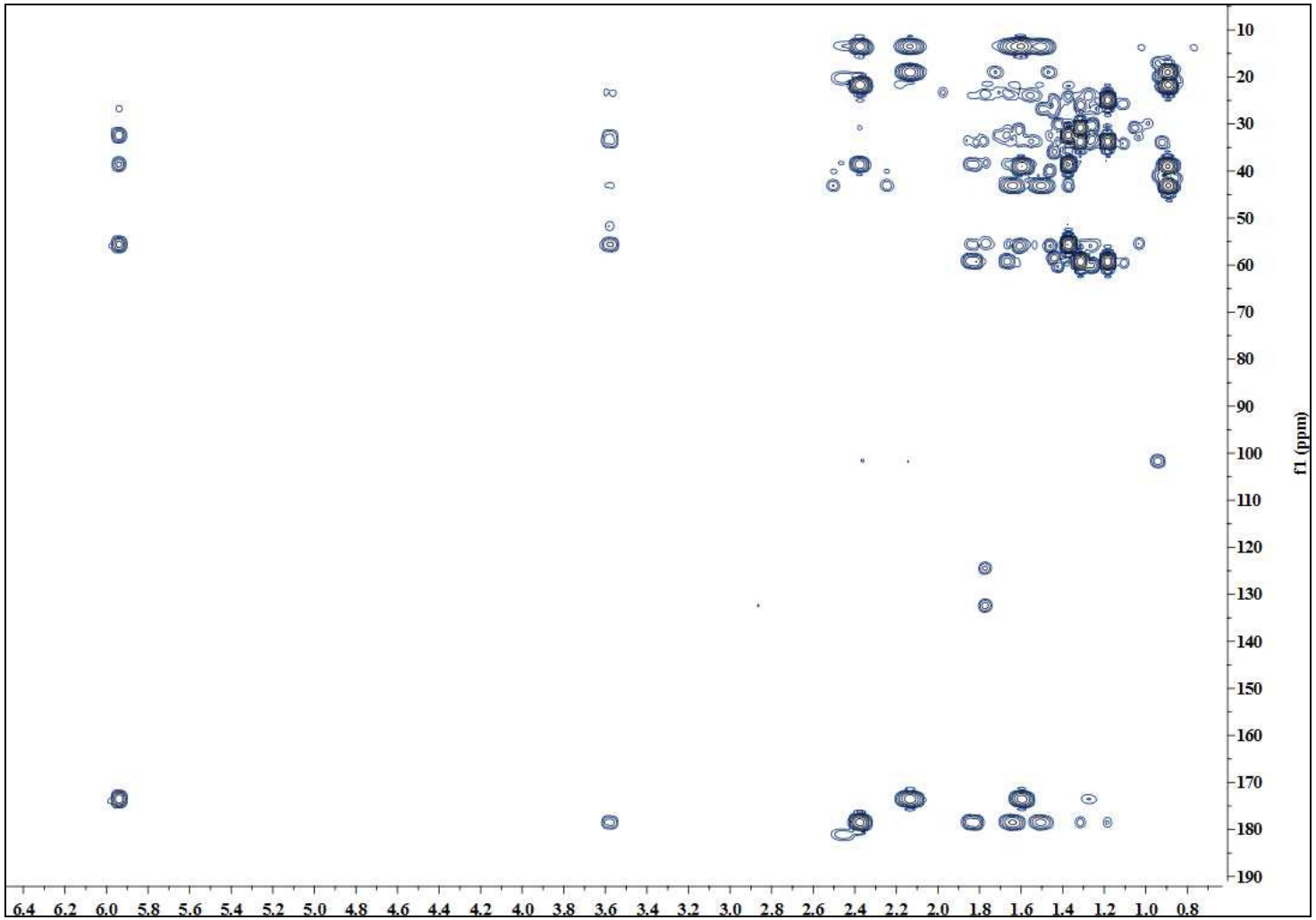
DEPT 90 ($CDCl_3$) spectrum of **4**



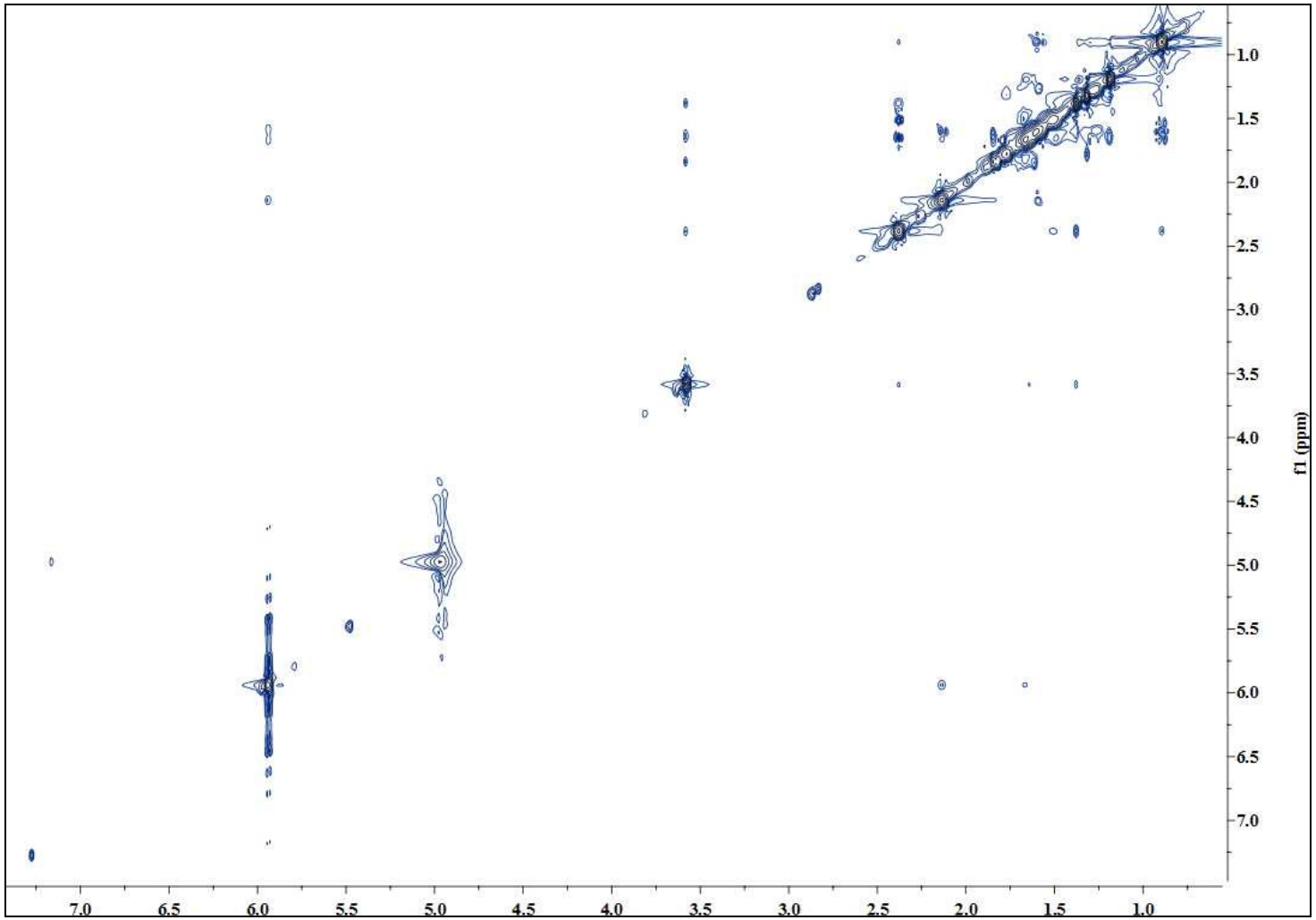
COSY ($CDCl_3$) spectrum of **4**



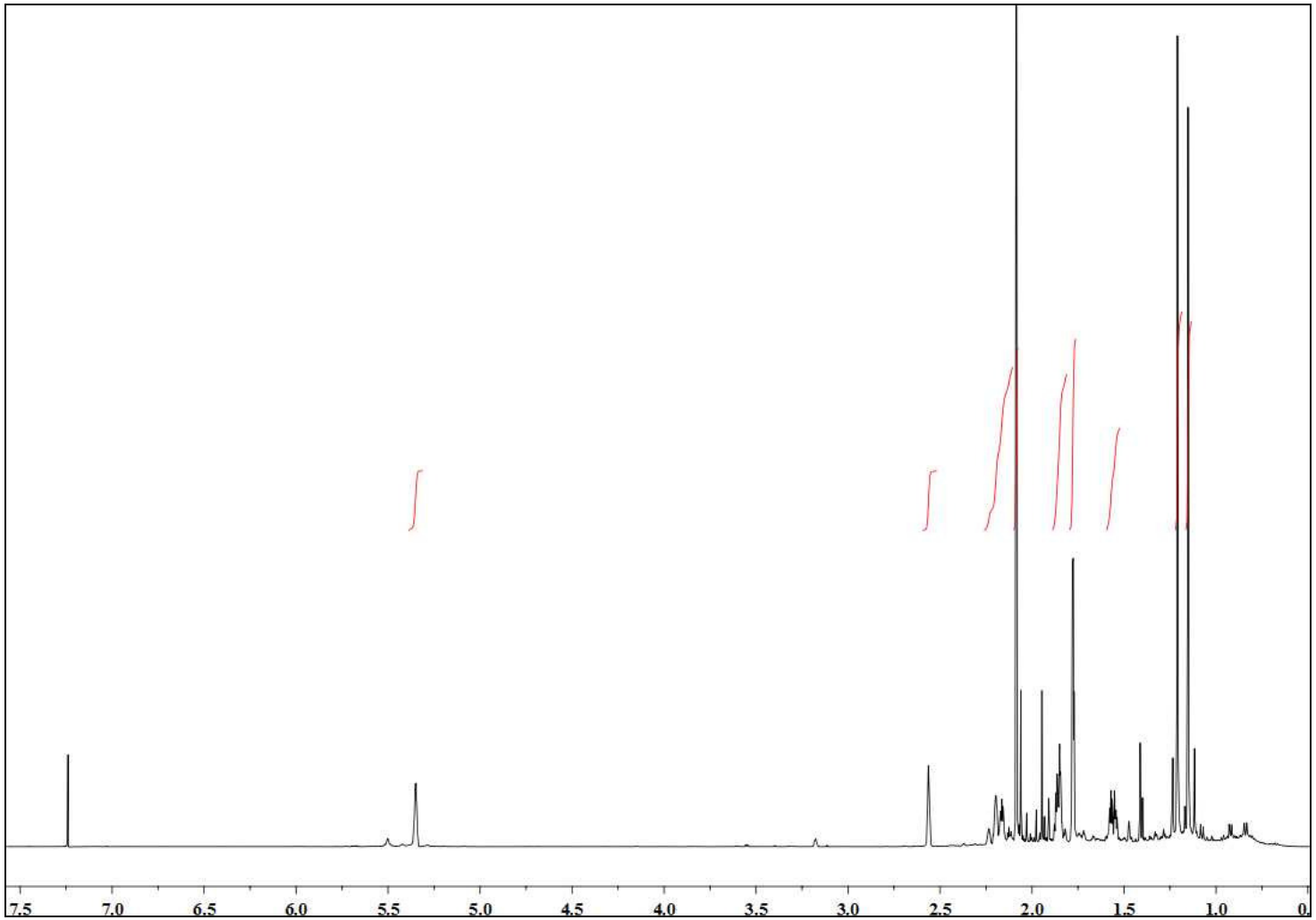
HMQC ($CDCl_3$) spectrum of **4**



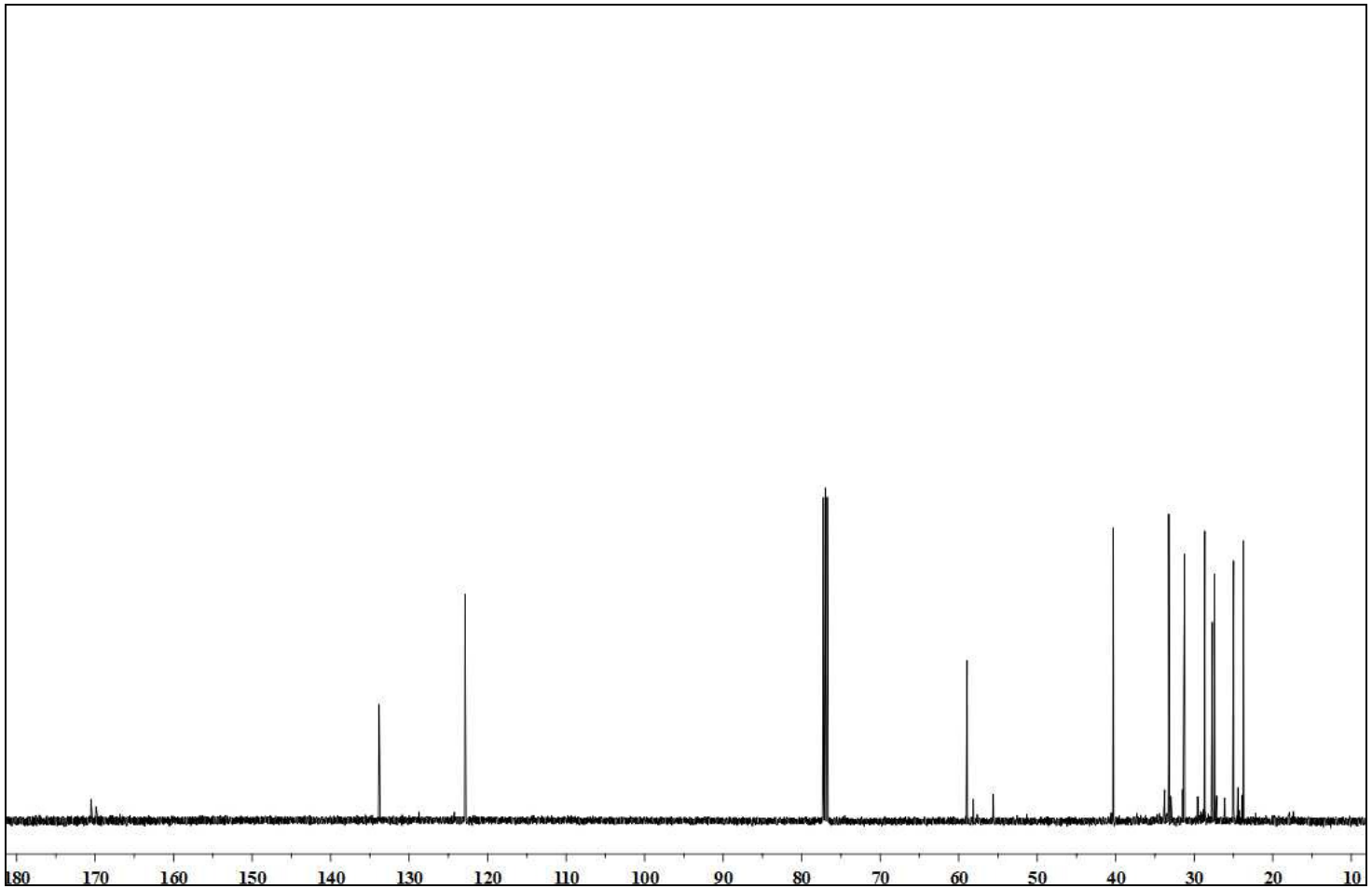
HMBC ($CDCl_3$) spectrum of **4**



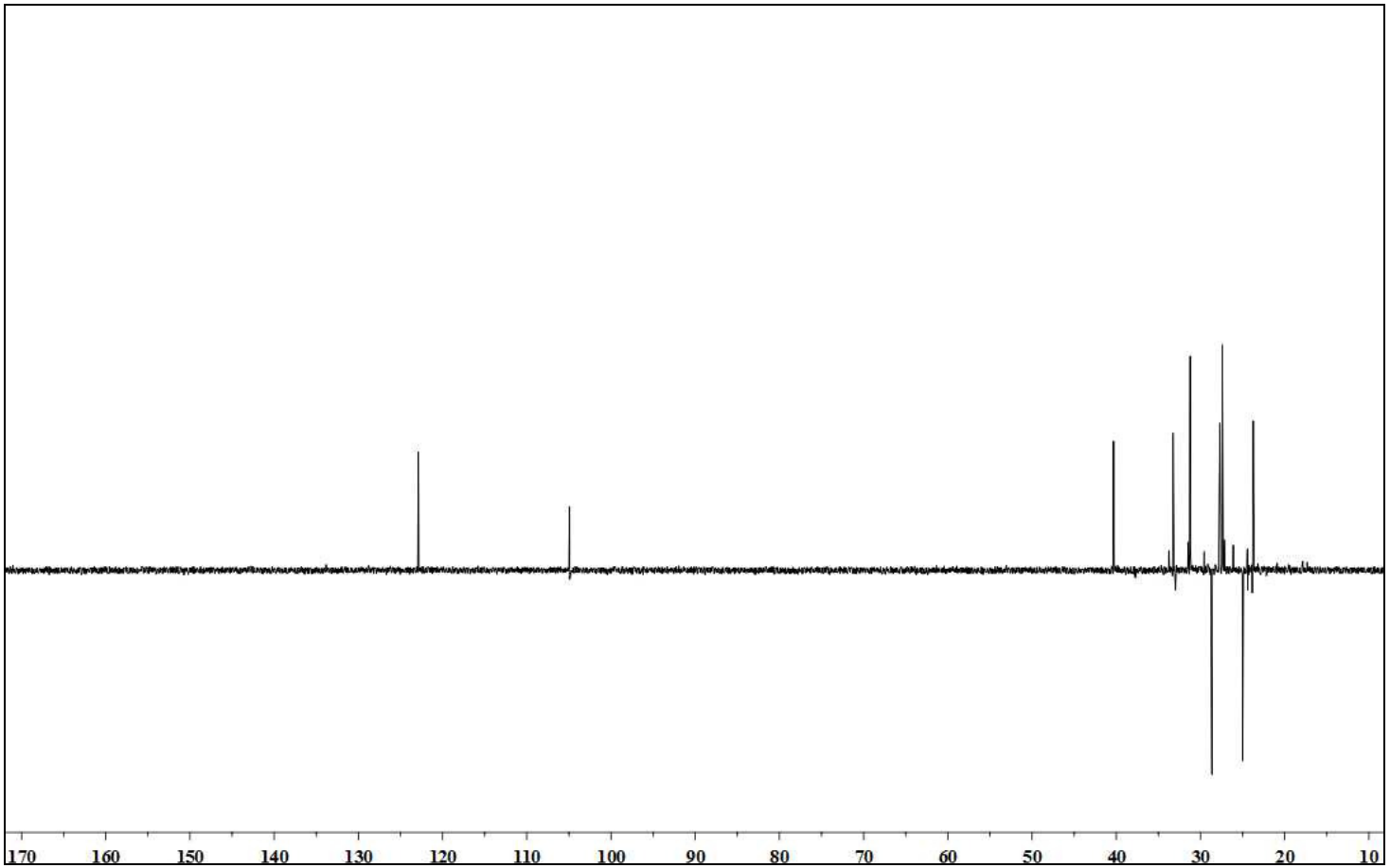
NOESY ($CDCl_3$) spectrum of **4**



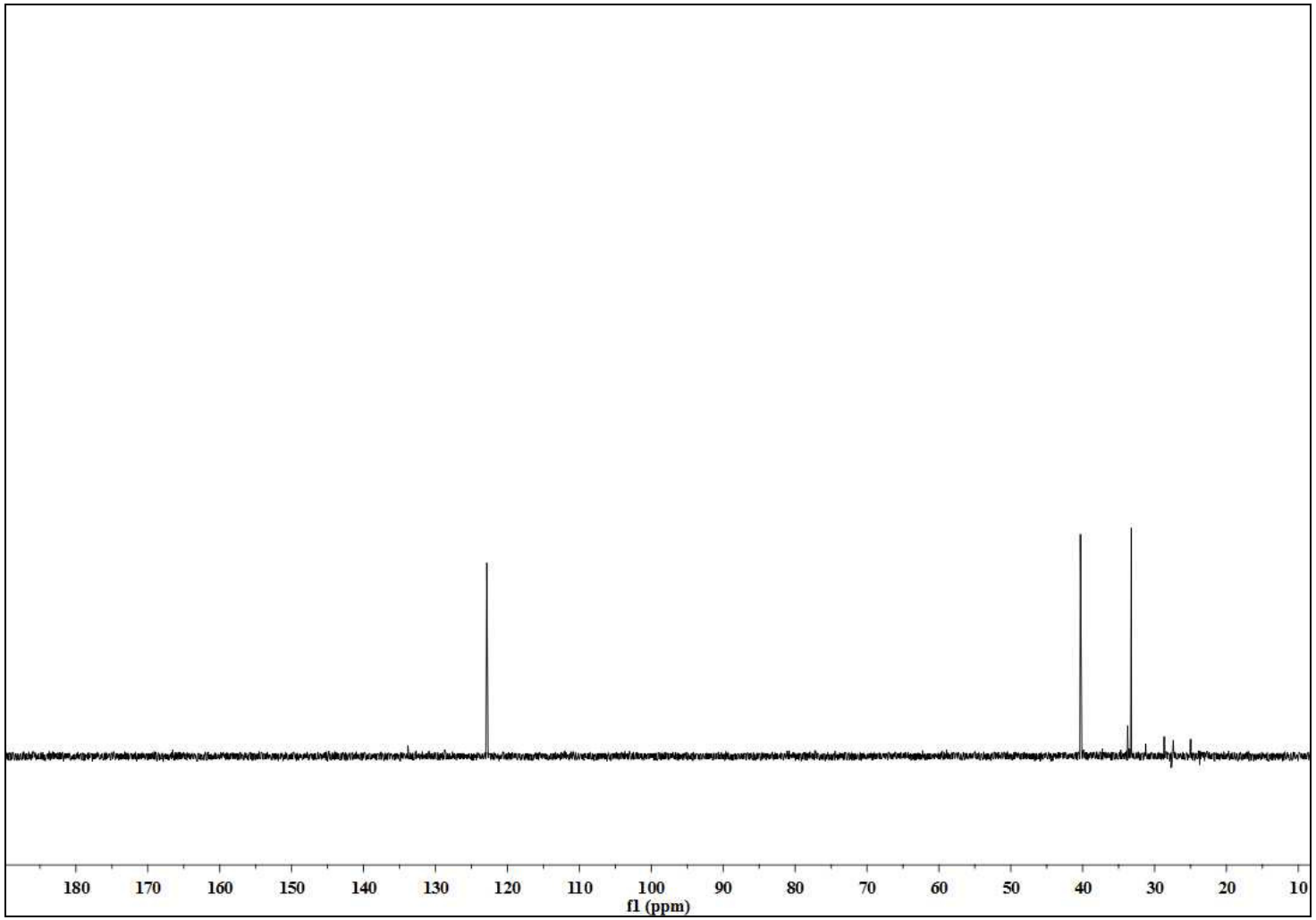
^{1}H-NMR spectrum of **5** (500 MHz, $CDCl_3$)



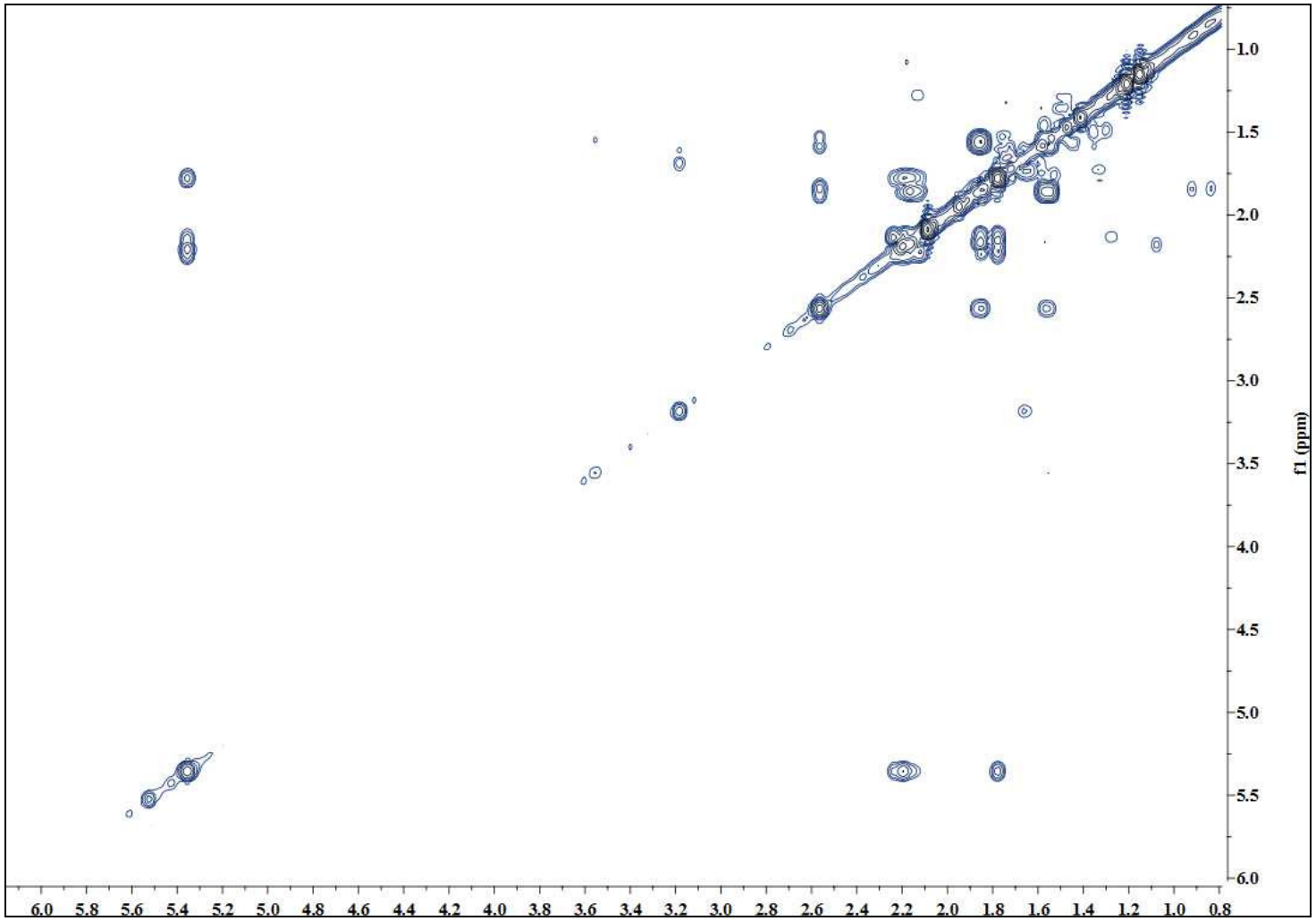
^{13}C-NMR spectrum of **5** (125 MHz, $CDCl_3$)



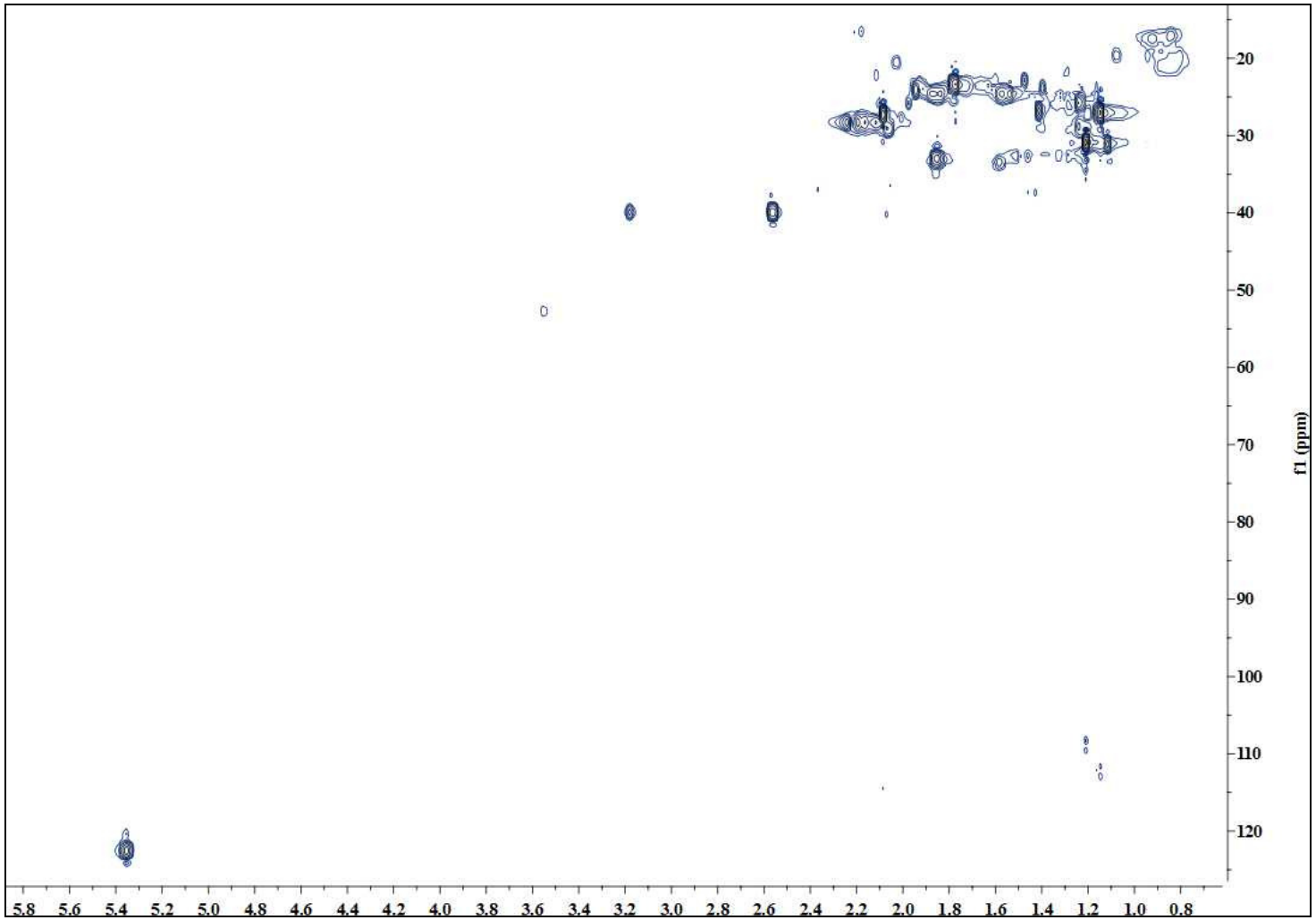
DEPT 135 ($CDCl_3$) spectrum of **5**



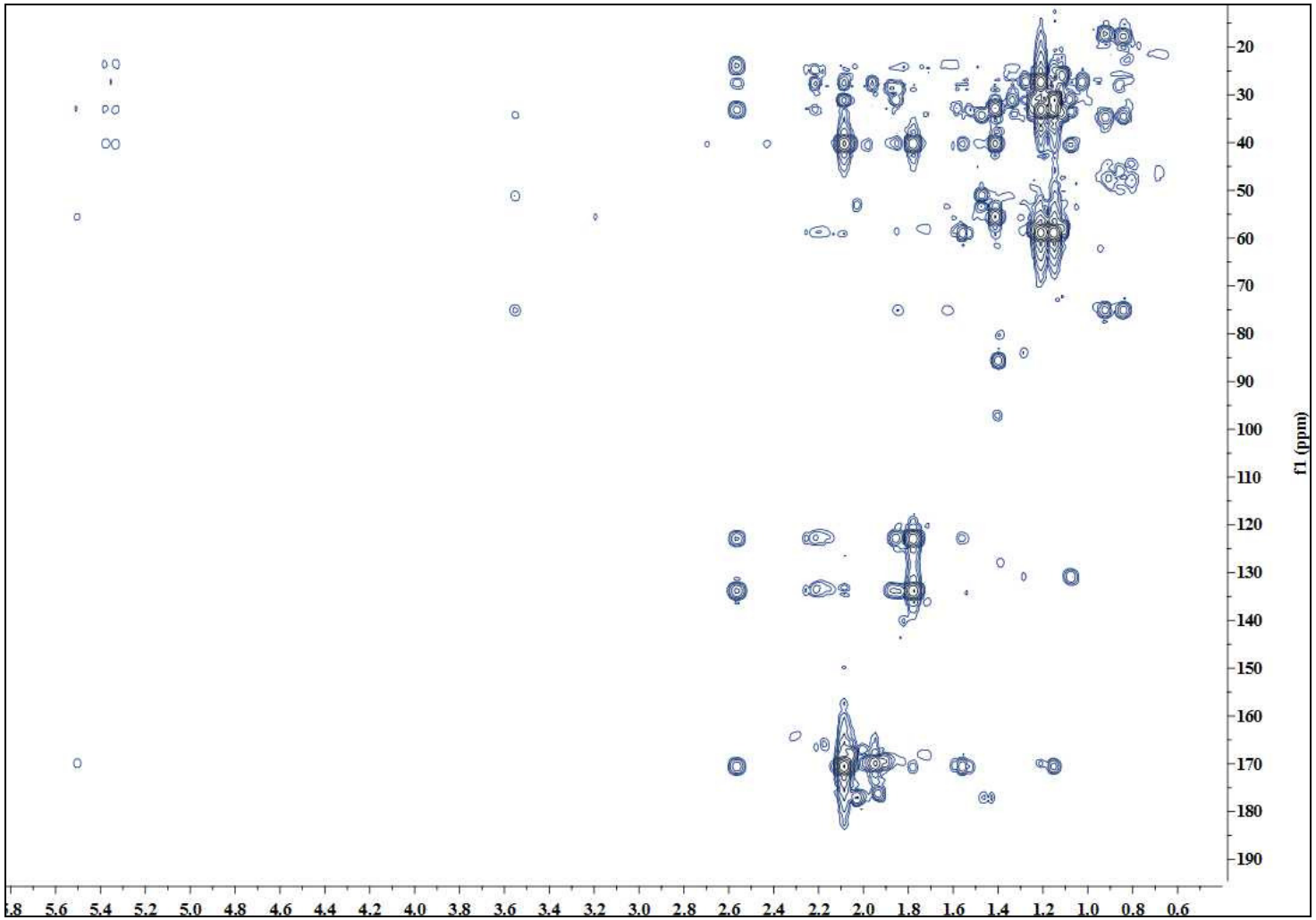
DEPT 90 ($CDCl_3$) spectrum of **5**



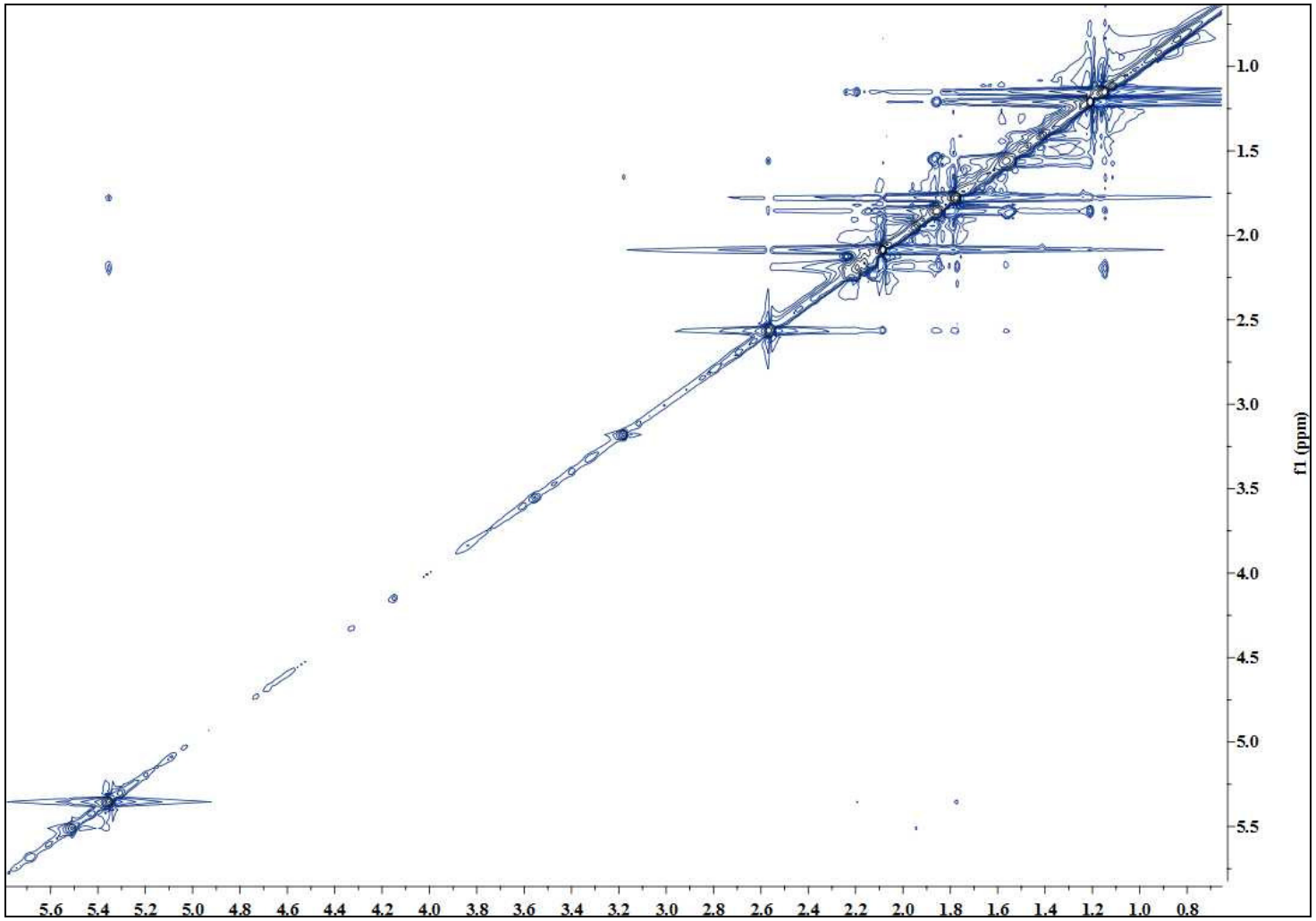
COSY ($CDCl_3$) spectrum of **5**



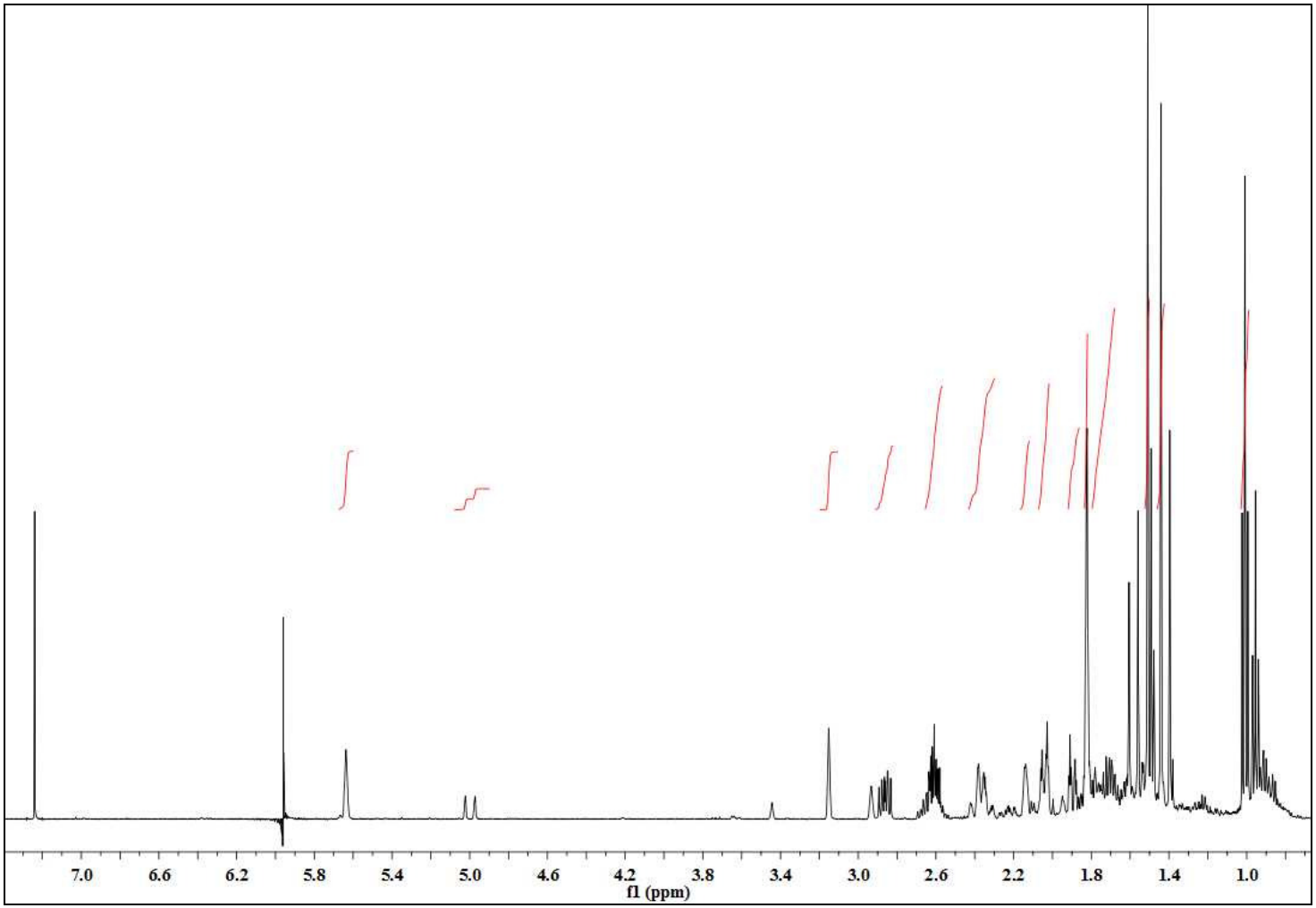
HMQC ($CDCl_3$) spectrum of **5**



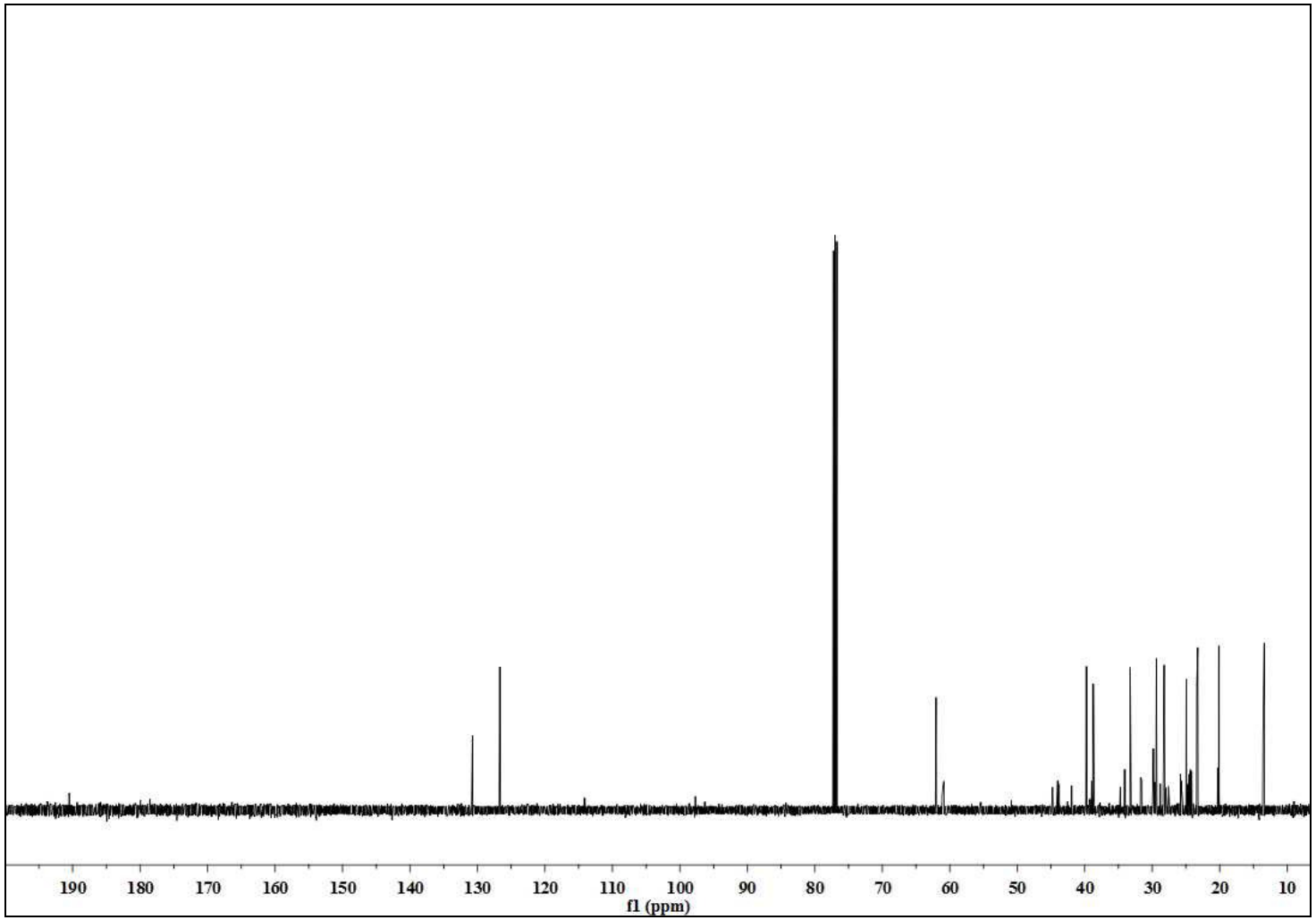
HMBC ($CDCl_3$) spectrum of **5**



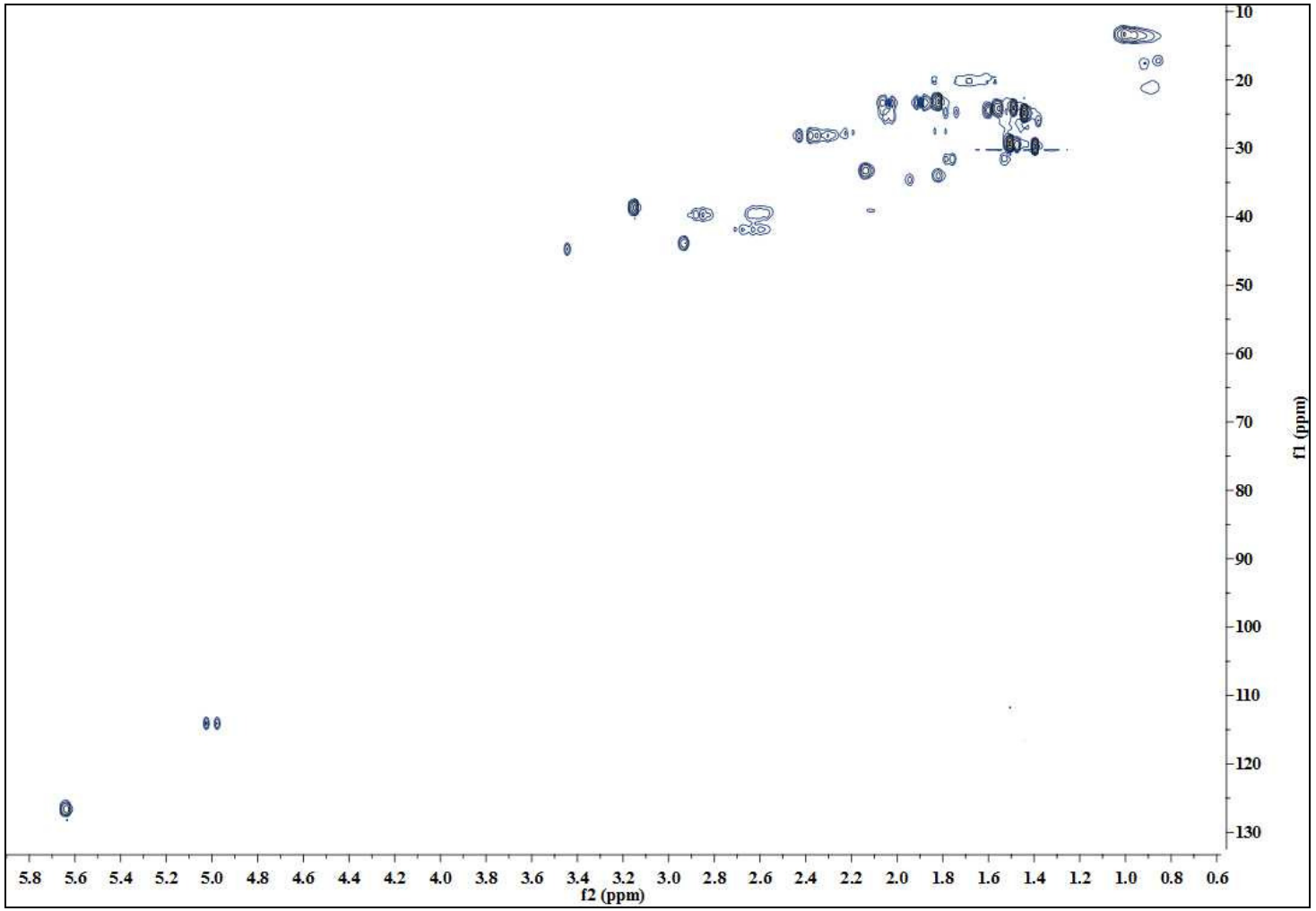
NOESY ($CDCl_3$) spectrum of **5**



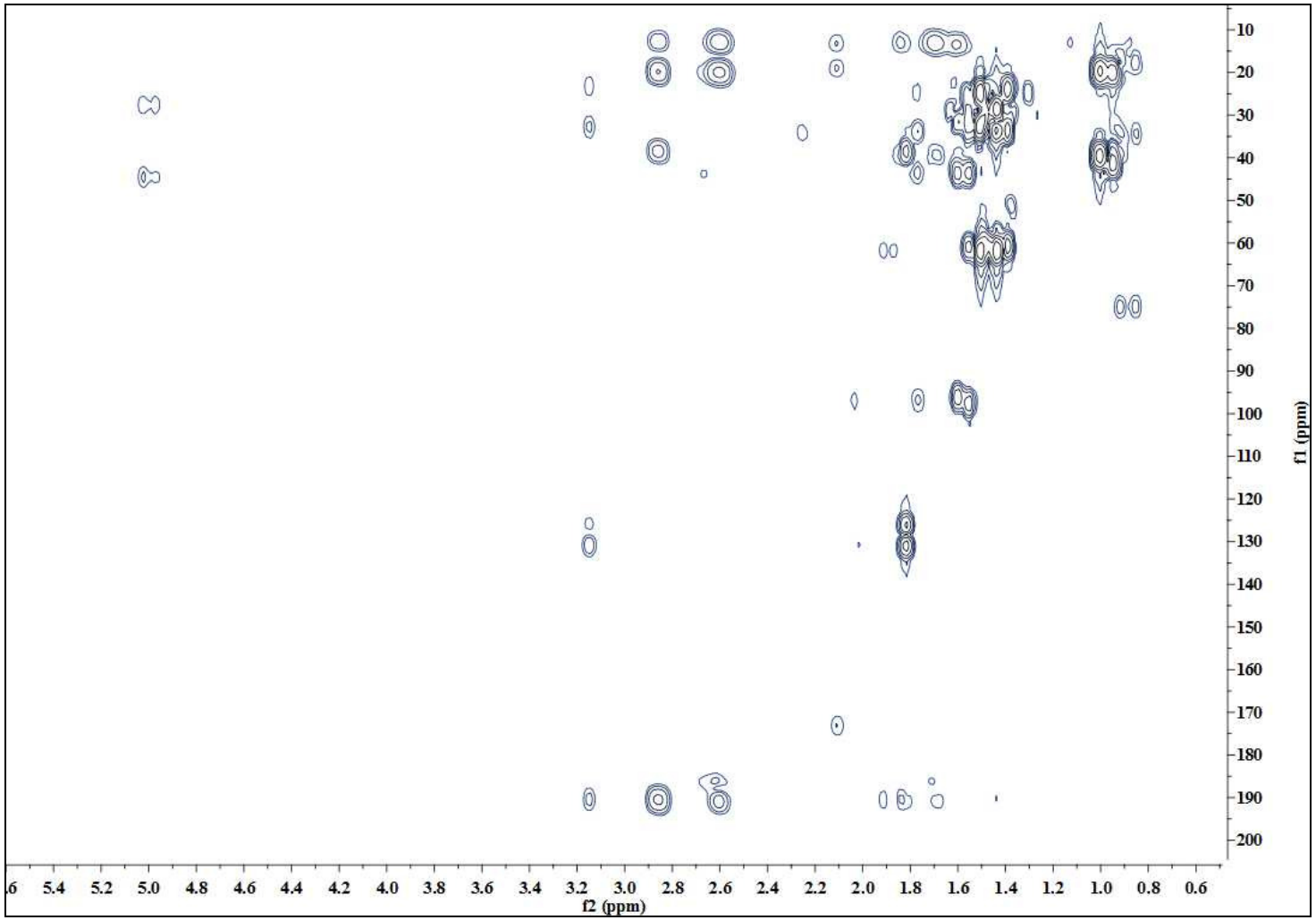
^{1}H-NMR spectrum of **6** (500 MHz, $CDCl_3$)



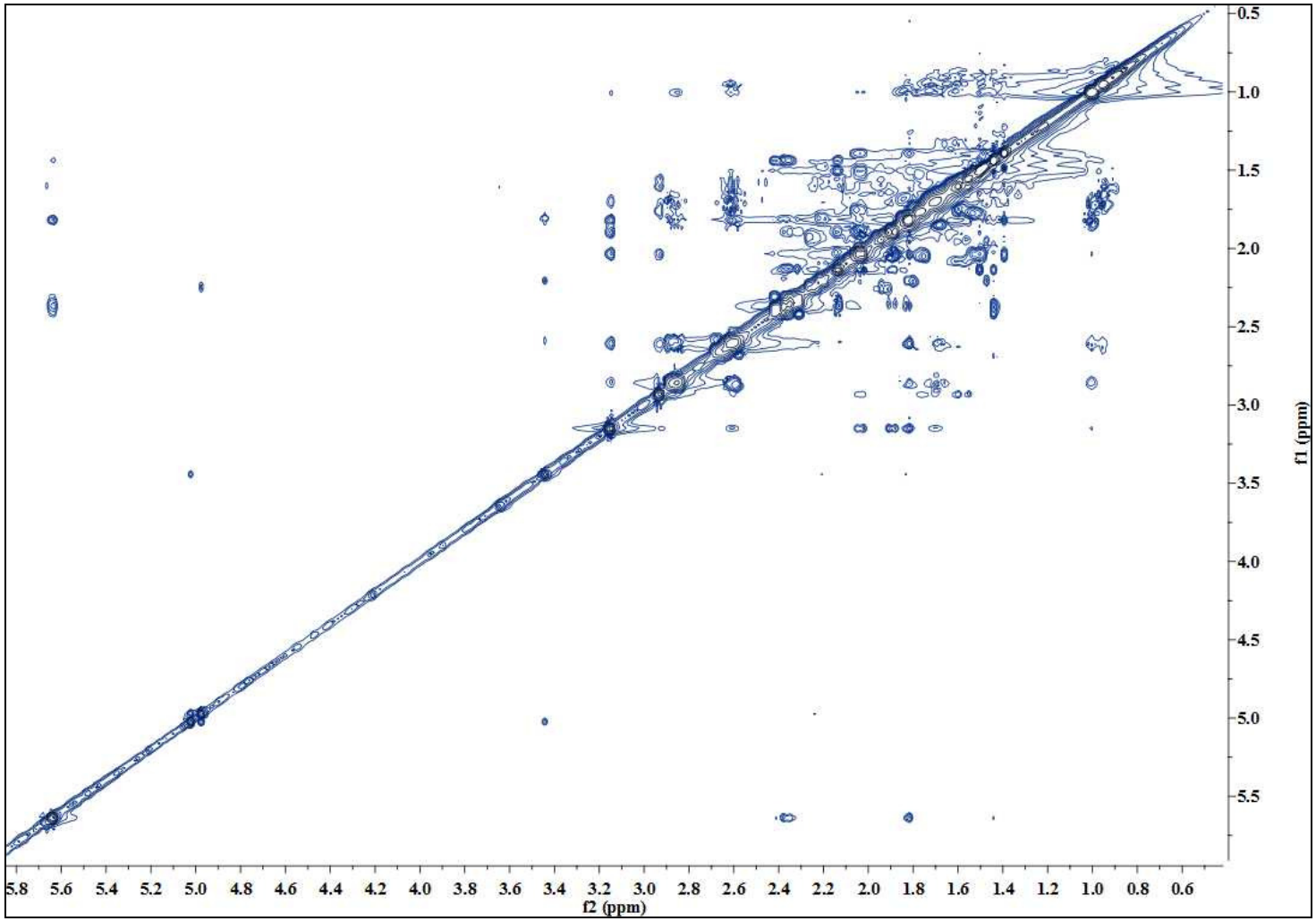
^{13}C-NMR spectrum of **6** (125 MHz, $CDCl_3$)



HMQC ($CDCl_3$) spectrum of **6**



HMBC ($CDCl_3$) spectrum of **6**



NOESY ($CDCl_3$) spectrum of **6**

VITAE

Name Mr. Sunan Jaisamut

Student ID 5110730012

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Second Class Honors) (Chemistry)	Suratthani Rajabhat University, Surat Thani, Thailand	2004
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Scholarship Awards during Enrolment

Scholarship was awarded by the Office of the Higher Education Commission, Thailand for supporting by grant fund under the program Strategic Scholarships for Frontier Research Network for the Ph.D. Program Thai Doctoral degree for this research and the Graduate School, Prince of Songkla University.

Lists of Publication and Proceeding

Publications

Jaisamut, S.; Hannongbua, S.; Prabpai, S.; Tancharoen, C.; Kongsaeree, P.; Yuenyongsawad, S.; Plubrukarn, A. Bridged tricyclic sesquiterpenes from the tubercle nudibranch *Phyllidia coelestis* Bergh. *J. Nat. Prod*. **2013**, *76*, 2158–2161.

Proceeding: International conferences

Jaisamut, S.; Plubrukarn, A. Novel sesquiterpene formamide from the nudibranch *Phyllidia* sp. Commission on Higher Education Congress IV: University Staff Development Consortium CHE-USDC Congress IV. The Zign Hotel, Pattaya, Chonburi, Thailand. 14-16 September 2011. (Poster)

Jaisamut, S.; Hannongbua, S.; Tancharoen, C.; Yuenyongsawad, S.; Kongsaeree, P.; Plubrukarn, A. Sesquiterpene formamide from the nudibranch *Phyllidia coelestis* Bergh. International Conference on Natural Products 2013 (ICNP 2013), Shah Alam, Selangor, Malaysia. 4-6 March 2013. (Poster)

Jaisamut, S.; Plubrukarn, A. Novel sesquiterpene formamide from the nudibranch *Phyllidia* sp. The 2nd current drug development international conference. Phuket Graceland resort & spa, Phuket, Thailand. 2-4 May 2511. (Full Proceedings, Poster)