Bioactive Constituents from the Sponge *Stylissa* cf. *massa* and the Soft Coral *Eleutherobia* sp.

Naphatson Chanthathamrongsiri

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy Program in Pharmaceutical Sciences

Prince of Songkla University

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This is to certify that the work here submitted is the result of the candidate's own investigations. Due acknowledgement has been made of any assistance received.

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(Miss Naphatson Chanthathamrongsiri)

Candidate

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

(Miss Naphatson Chanthathamrongsiri)

Candidate

ชื่อวิทยานิพนธ์ สารที่มีฤทธิ์ทางชีวภาพจากฟองน้ำ *Stylissa* cf. *massa* และปะการังอ่อน *Eleutherobia* sp.

ผู้เขียน นางสาว นภัสสร ฉันทธำรงศิริ

สาขาวิชา เภสัชศาสตร์

ปีการศึกษา 2556

บทคัดย่อ

การศึกษาสารประกอบทางเคมีที่มีฤทธิ์ต้านมาลาเรีย จากฟองน้ำ *Stylissa* cf. *massa* พบว่าสามารถแยกสารชนิดใหม่ในกลุ่มแอมฟิแลกเทนไดเทอร์ปีน ได้สองชนิด คือ (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-8-isocyanato-15-formamidoamphilect-11(20)-ene (**A**) และ (1*S**,3*S**,4*R**,7*S**,8*R**,12*S**,13*S**)-8-isothiocyanato-15-formamidoamphilect-11(20)-ene (**B**) รวมถึงสารที่ได้มีรายงานการแยกสกัดมาแล้วอีกสองชนิด คือ (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-8-isocyano-15-formamidoamphilect-11(20)-ene (**C**), และ (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-7-formamidoamphilecta-11(20),15-diene (**D**) โดยสาร **A** - **C** แสดงฤทธิ์ต้านมาลาเรียในระดับปานกลาง (IC_{50} = 0.52 - 0.85 μM) และไม่แสดงความเป็นพิษต่อเซลล์ MCF-7 (human breast adenocarcinoma).

การศึกษาสารประกอบทางเคมีของปะการังอ่อน *Eleutherobia* sp. สามารถแยกสารบริสุทธิ์ได้จำนวนสามชนิด คือ xeniolide A (**E**), thymine (**F**), และ 2*H*,5*H*,7*H*,9*H*-9-hydroxy-imidazole[1,5-*a*]pyridine-1,3-dione (**G**) สารทั้งสามชนิดแสดงความเป็นพิษต่อเซลล์ KB (human oral epidermoid carcinoma) และเซลล์ HeLa (human cervical carcinoma) ในระดับปานกลาง (IC_{50} = 0.17 - 0.84 μM).

การศึกษาการเกิดสารประกอบเชิงซ้อน ระหว่าง 8,15-diisocyanoamphilecta-11-(20)-ene (DIA) กับฮีม ด้วยเทคนิคทางสเปคโทรสโกปี และสเปคโทรเมทรี ได้แก่ UV-visible absorption, emission, CD, ^{1}H NMR, และ ESIMS โดยสามารถสรุปได้ว่า DIA สามารถเกิดสารประกอบเชิงซ้อนกับฮีม ด้วยอัตราส่วนระหว่างฮีม และ DIA เท่ากับ 1:1 และ 1:2 ในสารละลาย 50% น้ำ และ DMSO และจากการผลการศึกษาพบว่า DIA ไม่สามารถเกิดสารประกอบเชิงซ้อนกับฮีโมโกลบินได้

Thesis Title Bioactive Compounds from the Sponge *Stylissa* cf. *massa* and the Soft Coral *Eleutherobia* sp.

Author Miss Naphatson Chanthathamrongsiri

Major Program Pharmaceutical Sciences

Academic year 2013

ABSTRACT

The chemical investigations of the antiplasmodial agents from the sponge *Stylissa* cf. *massa* led to isolation of two new amphilectane diterpenes, (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-8-isocyanato-15-formamidoamphilect-11(20)-ene (**A**), and (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-8-isothiocyanato-15-formamidoamphilect-11(20)-ene (**B**), and two of known compounds, (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-8-isocyano-15-formamidoamphilect-11(20)-ene (**C**), and (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-7-formamidoamphilecta-11(20),15-diene (**D**). Compounds **A** - **C** showed the moderate antiplasmodial activity (IC_{50} = 0.52 - 0.85 μM) but did not have cytotoxic activity to MCF-7 cells (human breast adenocarcinoma).

The chemical investigation of the soft coral *Eleutherobia* sp. have yield three known compounds, xeniolide A (**E**), thymine (**F**), and 2*H*,5*H*,7*H*,9*H*-9-hydroxy-imidazole[1,5-*a*] pyridine-1,3-dione (**G**). All of the isolated compounds showed a moderate cytotoxicity against KB (human oral epidermoid carcinoma) and HeLa (human cervical carcinoma) (IC_{50} = 0.17 - 0.84 μM).

The complex characterization of heme and 8,15-diisocyanoamphilecta-11(20)-ene (DIA) using spectroscopy or spectrometry methods, namely UV-visible absorption, emission, CD, ^{1}H NMR, and ESIMS, suggested that DIA can form complex with heme with both 1:1 and 1:2 ratios, but cannot form complex with hemoglobin.

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Naphatson Chanthathamrongsiri

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LIST OF ABBREVIATIONS AND SYMBOLS

$[\alpha]_D$	specific rotation
δ	chemical shift (in ppm)
ε	molar extinction coefficient
λ_{max}	maximum wavelength
ν_{max}	maximum wave number
br	broad (for NMR signals)
c	concentration
COSY	correlation spectroscopy
d	doublet (for NMR signals)
EIMS	electron-impact mass spectroscopy
ESIMS	electro-sprayed ionization mass spectroscopy
EMEM	Earle's salt minimum essential medium
HEPES	N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid
HMBC	heteronuclear multiple-bond multiple-quantum coherence
HMQC	heteronuclear multiple-quantum coherence
HPLC	high performance liquid chromatography
HREIMS	high-resolution electron-impact mass spectroscopy
HRESIMS	high-resolution electro-sprayed ionization mass spectroscopy
IC_{50}	inhibitory concentration at 50% of tested subject
IR	infrared
J	coupling constant
m/z	mass-over-charge ratio
MS	mass spectrometry
mult	multiplicity

LIST OF ABBREVIATIONS AND SYMBOLS

NMR	nuclear magnetic resonance
nOe-ds	nuclear Overhauser effect-difference spectrum
RPMI	Roswell Park Memorial Institute medium
s	singlet (for NMR signals)
SRB	sulphorhodamine B
t	triplet (for NMR signals)
t_R	retention time
TLC	thin layer chromatography
UV	ultraviolet

CHAPTER 1

INTRODUCTION

The natural products have been the most important sources for bioactive compounds in drug discovery. Whereas terrestrial plants are among the most common and widely explored, other sources, including animals and microrganisms - both aquatic and terrestrial ones, are as well alternative among natural products chemists. With specific niche that drives each species to survive in a specific environment, physical appearances have led to different chemicals production in each species. Specifically for marine organisms, the unique marine environments are the major driving forces that yield different metabolisms among marine animals. Additionally, with the sedentary and shell-less characteristics, such marine animals therefore have evolved with the ability to produce their own chemical warfares for their protection from the predators. Combined with the fact that the vast area of the oceans were more than 70% of the earth surface, providing more than 95% of living organisms as marine associated lives, the oceans are clearly the main sources of natural products to be exploited in many ways.

1.1 Drugs derived from marine natural products

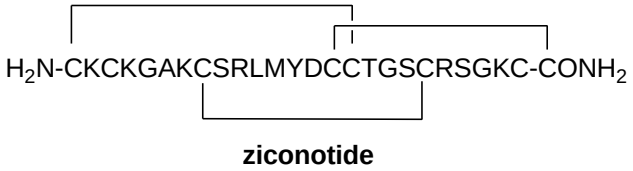
To date, there are only a handful of medicines, either derived directly or partially from marine natural products, that are approved by US FDA and EMEA, and are available commercially. Among these, arabinonucleosides, including cytarabine and vidarabine, which are inspired by spongothymidine from sponge *Cryptotethia crypta* (*Tethya crypta*) could be considered the first and the most long-lasting class of marine-derived medicines widely used in clinic. Cytarabine or ara-C (Cytosar-U®, DepoCyt®) was approved by US FDA as an antileukemic agent in 1969. The synthetic purine analog of spongouridine, vidarabine or ara-A (Vira-A®) as an antiviral agent for herpes, vaccinia, and varicella zoster virus (Newman and Cragg, 2004, Mayer et al, 2010). Another anticancer drug, trabectedin or ecteinascidin-743

(Yondelis®), was first isolated from the tunicate, *Ecteinascidia turbinata*. Trabectedin was approved in European Union in 2007 for metastatic soft tissue carcinoma treatment, and in 2009 for relapsed platinum-sensitive ovarian cancer (Mayer et al, 2010; Indumathy and Dass, 2013).

Eribulin mesylate (halichondrin E7389; Halaven®) was derived from halichondrin B, first isolated from the sponge *Halichondria okadai*. Eribulin mesylate was aprroved by US FDA in 2010 for the treatment of breast cancer in late-stage (Mayer et al, 2010; Indumathy and Dass, 2013). Very recently, brentuximab vedotin (SGN-35; Adcetris®), the monomethyl auristatin E conjugated with anibody, was approved by US FDA in 2011 as anticancer drug for the treatment of Hodgkin's lymphoma and anaplastic large cell lymphoma. The drug is in fact derived from dolastatin 10, first isolated from sea hare *Dolabella auricularia* (Senter and Sievers, 2012; Trail, 2013; Newman and Cragg, 2014).

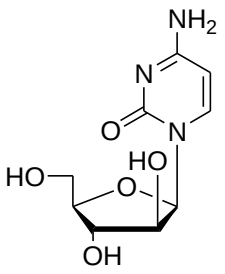
Apart from anticancer drugs, a few marine-derived medicines used in other disease areas have also been developed. Ziconotide or ω-conotoxin MVIIA (Prialt®) was approved by US FDA in 2004 as an analgesic agent used in severe chronic pain treatment for cancer or AIDS-related patients. ω-Conotoxin MVIIA was first isolated from the venom of cone snail, *Conus magus* (Mayer et al, 2010). The ω-3 acid ethyl ester, the polyunsaturated fatty acid (PUFA; Lovaza®), from marine fish was approved by US FDA in 2004 for hypertriglyceridemia (Tur et al, 2012; GlaxoSmithKline, 2014).

In addition to those compounds that have been approved and available commercially, several marine-derived compounds are currently in their clinical trials. Some examples that are in the advanced states and are potentially becoming available in clinic are shown in Table 1.

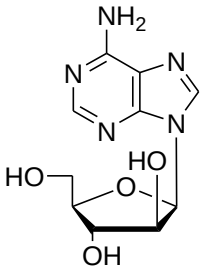


ziconotide

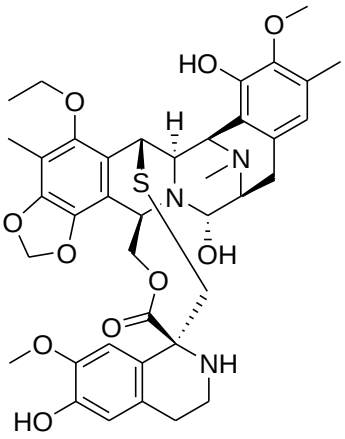
Figure 1. Drugs in clinical used from marine natural products



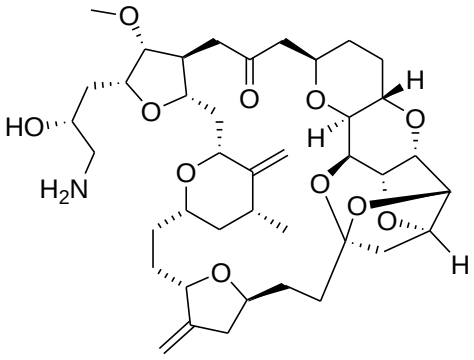
cytarabine



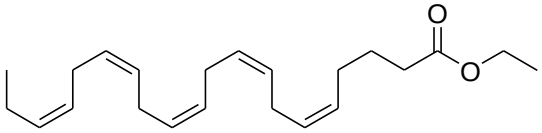
vidarabine



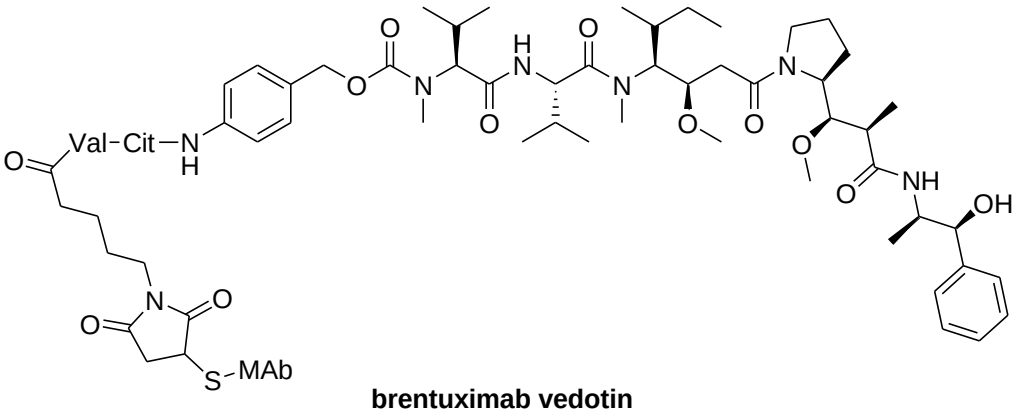
trabecidin



eribulin mesylate



ω–3–acid ethyl esters



brentuximab vedotin

Figure 1. Drugs in clinical used from marine natural products (cont.)

Table 1. Bioactive compounds derived from marine organisms in clinical trials

Compound	Source	Clinical use	Status
plitidepsin (Aplidin®; analog of didemnin B; cyclic depsipeptide)	*Aplidium albican* (tunicate)	multiple myeloma	Phase III (Mayer et al, 2010; Mayer, 2014; Indumathy and Dass, 2013)
tetrodotoxin (Tectin®; alkaloid)	Tetraodontidae puffer (fish)	pain related to cancer	Phase III (Mayer, 2014; Narahashi, 2008; Newman and Cragg, 2014)
soblidotin (TZT-1027; analog of dolastatin 10; peptide)	*Dolabella auricularia* (sea hare)	lung cancer and soft tissue sarcroma	Phase II (Mayer, 2014, Noro et al, 2012)
3-(2,4-dimethoxybenzy-lidene)-anabaseine (DMXBA, GTS-21; analog of anabaseine; alkaloid)	*Amphiporus lactifloreus* (nematide worms)	alzheimer and schizophrenia	Phase II (Mayer et al, 2010; Mayer, 2014)
PM00104 (Zalypsis®; analog of jorumycin; alkaloid)	*Jorunna funebris* (nudibranch)	cervical cancer	Phase II (Mayer, et al, 2010; Mayer, 2014; Indumathy and Dass, 2013)
lurbinectedin (PM01183; analog of trabectedin; alkaloid)	*Ecteinascidia turbinata* (tunicate)	advance solid tumors	Phase II (Mayer, 2014; Newman and Cragg, 2014; Leal et al, 2010)

Table 1. (cont.)

Compound	Source	Clinical use	Status
glembatumumab vedotin (CDX-011; antibody conjugated with monomethylauristatin E)	*Symploca* sp. (cyanobacteria)	breast cancer	Phase II (Mayer, 2014; Newman and Cragg, 2014)
kahalalide F (PM-92012; cyclic depsipeptide)	*Elysia rufescens* (mollusk)	non-small cell lung cancer	Phase II (Mayer et al, 2010; Newman and Cragg, 2014)
squalamine (amino steroid)	*Squalus acanthias* (shark)	retinal neovascularization	Phase II (Noro et al, 2012)
KRN-7000 (glycosphingolide)	*Agelas mouritianus* (sponge)	chronic hepatitis B and C	Phase II (Noro et al, 2012)
salinosporamide A (marizomib, NPI-0052; bicyclic of lactam and lactone)	*Salinispora tropica* (actenomycetes)	multiple myeloma	Phase II (Mayer et al, 2010; Mayer, 2014; Indumathy and Dass, 2013)
tasidotin (Synthadotin, ILX-651; analog of dolastatin 15; peptide)	*Dolabella auricularia* (sea hare)	melanoma, prostate cancer, and non-small cell lung carcinoma	Phase II (Mayer, 2014; Noro et al, 2012)
pseudopterosins (diterpene glycoside)	*Pseudopterogorgia elisabethae* (soft coral)	wound healing	Phase II (Mayer et al, 2010)

Table 1. (cont.)

Compound	Source	Clinical use	Status
plinabulin (NPI-2358; analog of halimide; diketopiperazine)	*Aspergillus* sp. (fungus)	non-small cell lung carcinoma	Phase II (Mayer et al, 2010; Mayer, 2014)
PM060184 (polyketide)	*Lithoplocamia lithistoides* (sponge)	solid tumors	Phase I (Mayer, 2014; Newman and Cragg, 2014)
bryostatin 1 (polyketide)	*Bugula neritina* (bryozoan)	metastatic solid tumors	Phase I (Mayer et al, 2010; Mayer, 2014)
vorsetuzumab mafdotin (SGN-75; antibody conjugated with monomethylauristatin F; analog of dolastatin 10)	*Symploca* sp. (cyanobacteria)	non-Hodgkin's lymphoma and renal carcinoma	Phase I (Newman and Cragg, 2014)
ASG-5ME (antibody conjugated with monomethyl-auristatin E; analog of dolastatin 10)	*Symploca* sp. (cyanobacteria)	prostate, gastric, and pancreatic neoplasms	Phase I (Newman and Cragg, 2014; Trail et al, 2013)
hemiasterlin (E7974; tripeptide)	*Hemiasterella minor* (sponge)	malignant tumors	Phase I (Mayer et al, 2010; Newman and Cragg, 2014)

1.2 The sponge *Stylissa* spp. and the chemical constituents

The sponges of the genus *Stylissa* belong to family Dictyonellidae, order Halichondrida, class Demospongiae. To date, there have been at least 20 species identified. The sponges have been found distributing throughout South Africa, Asia and Australia (World Porifera Database, 2014).

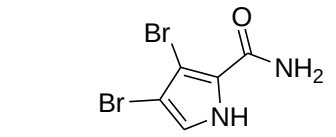
To date, up to 80 compounds have been isolated from the sponges of the genus *Stylissa*, particularly from *S. caribica*, *S. carteri*, *S. massa* and *S. flabellata*, and also from a handful of unidentified specimens of *Stylissa* spp. Roughly, the chemical constituents from *Stylissa* sponges can be categorized into three major classes; pyrrole carboxylic acid-derived and related alkaloids, cyclic peptides, and terpenes bearing isonitrile and related functionalities. Compounds with other structural genres, including steroids and glycolipids have also been reported.

Since the isonitrile terpenes and their derivatives are also a focal point in this dissertation, the review on this group of terpene compounds is omitted here and is discussed extensively in section 1.4.

1.2.1 Pyrrole carboxylic acids and derivatives

Presumably derived from proline, pyrrole carboxylic acid derivatives reported from most *Stylissa* sponges are generally brominated in an extended degree, from mono- to di-bromination, and the orientation can be found either in positions 2, or 3, or both. The structures can also be found in a wide range of complexity from a simple carbonamide as in 3,4-dibromopyrrole-2-carbonamide (**1**) (Fouad et al, 2012) and 4,5-dibromopyrrole-2-carbonamide (**2**) (Tasdemir et al, 2002) to coupled units with various amino acids, including histidine, as in oroidin (**3**) (Eder et al 1999; Tasdemir et al, 2002; Mohammed et al, 2006; Wang et al, 2014), lysine, as in 4-bromopyrrole-2-carboxy-*N*(ε)-lysine (**4**), arginine, as in 4-bromopyrrole-2-carboxyarginine (**5**) (Grube and Köck, 2006a), and aspatic acid as in aldisine (**6**) (Eder et al, 1999; Tasdemir et al, 2002; Grube and Köck, 2006a; Mohammed et al, 2006), hymenin (**7**) (Eder

et al, 1999; Tasdemir et al, 2002), stevensine (**8**) (Eder et al, 1999; Mohammed et al, 2006; Fouad et al, 2012; Wang et al, 2014), (*Z*)-hymenaldisine (**9**) (Eder et al, 1999; Tasdemir et al, 2002; Yamaguchi et al, 2013), and latonduine A (**10**) (Linington et al, 2003; Fouad et al, 2012). Intra- and intermolecular couplings also yield a more complicated, yet fascinating alkaloids as in dibromoisophakellin (**11**) (Assmann et al, 2001), sceptrin (**12**) (Eder et al, 1999; Tasdemir et al, 2002; Mohammed et al, 2006), ageliferin (**13) (**Assmann et al, 2001), stylissazole A (**14**) (Patel et al, 2010), massadine (**15**) (Nishimura et al, 2003; Buchanan et al, 2007a; Grube and Köck, 2007), stylissadine A (**16)** (Grube and Köck, 2006b; Buchanan et al, 2007b), and palau'amide (**17**) (Buchanan et al, 2007a; Buchanan et al, 2007b).



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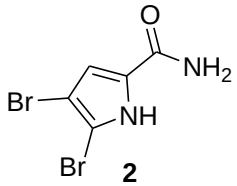

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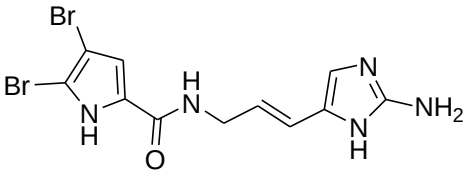

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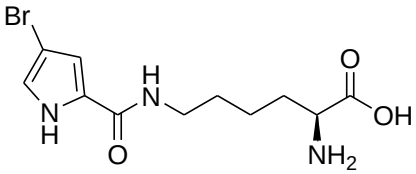

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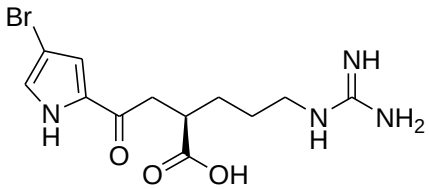

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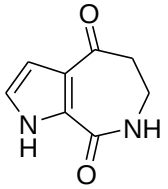

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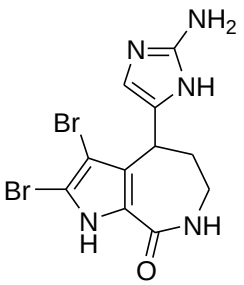

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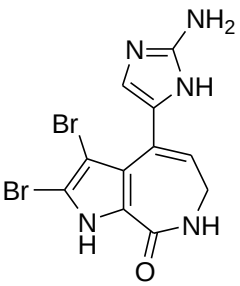

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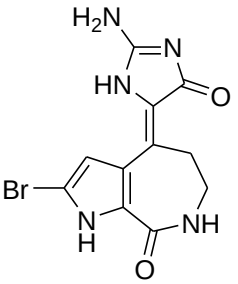

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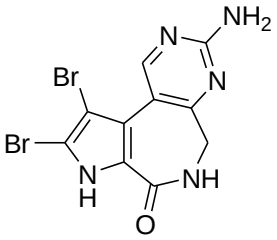

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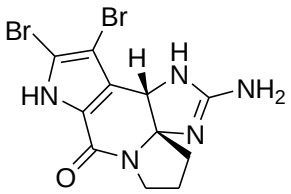

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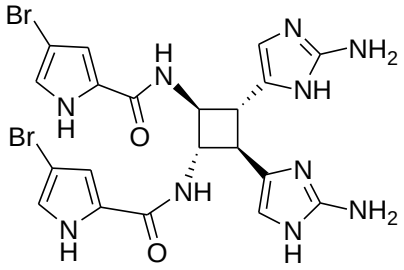

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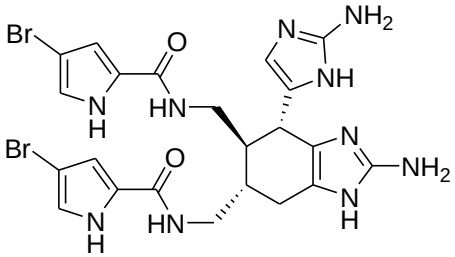

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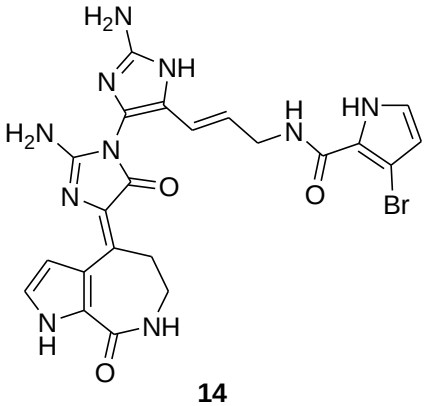

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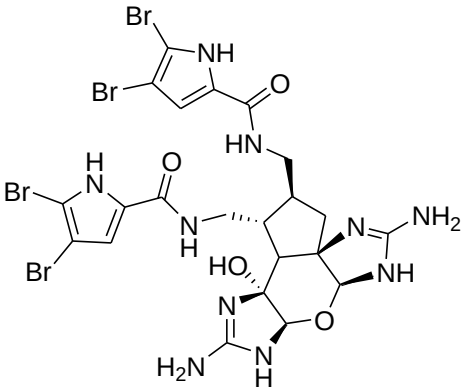

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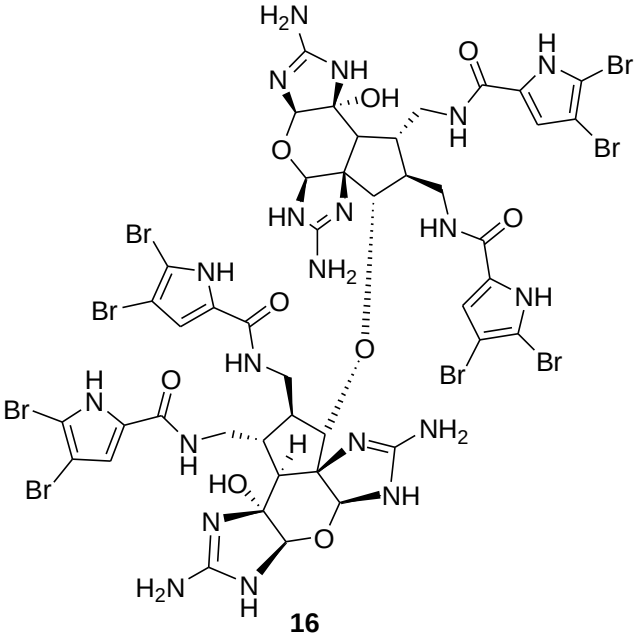

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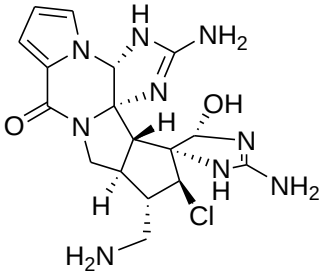

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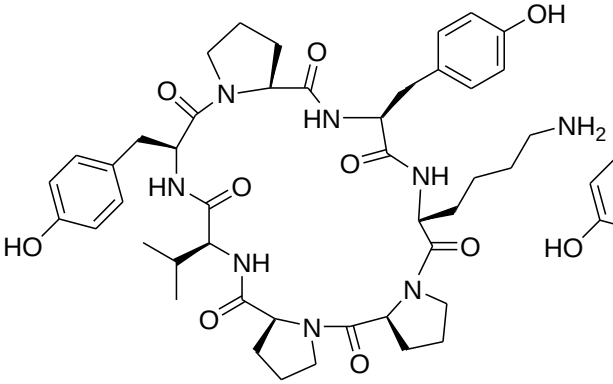
1.2.2 Cyclic peptides

Although far less frequent, cyclic peptides are another class of compounds isolated from *Stylissa* sponges. To date, there have been only 13 cyclic peptides reported from the sponges in this genus. They are stylissamides A-D (**18**-**21**) (Schmidt et al, 2007), stylissamides E and F (**22** and **23**) (Christine and Köck, 2010), and stylissamides G and H (**24** and **25**) (Wang et al, 2014) reported from *S. caribica*, stylissamide X (**26**) from *Stylissa* sp. (Arai et al, 2012), stylisins 1 (**27**) and 2 (**28**) from *S. caribica* (Mohammed et al, 2006; Wang et al, 2014), phakellistatin 13 (**29**) from *S. caribica* (Mohammed et al, 2006), and stylissatin A (**30**) from *S. massa* (Kita et al, 2013). Noticeably, most of the peptide reviewed here are heptapeptide, excepted for stylissamide X, which is octapeptide.



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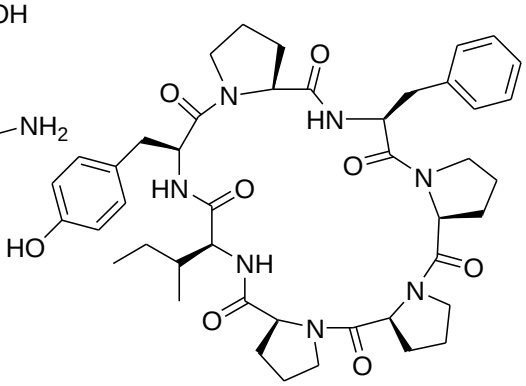

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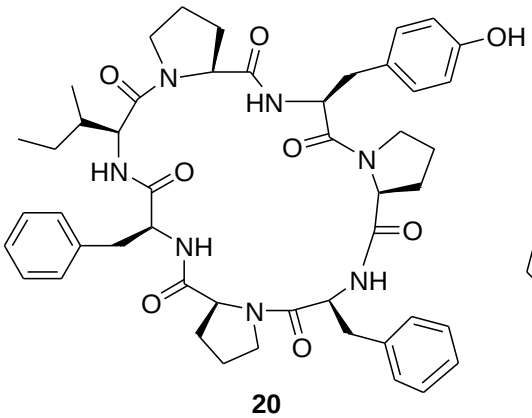

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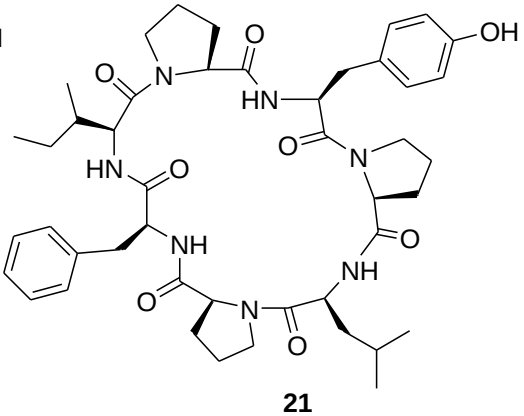

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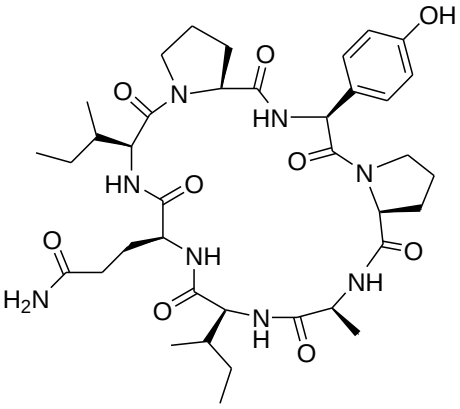

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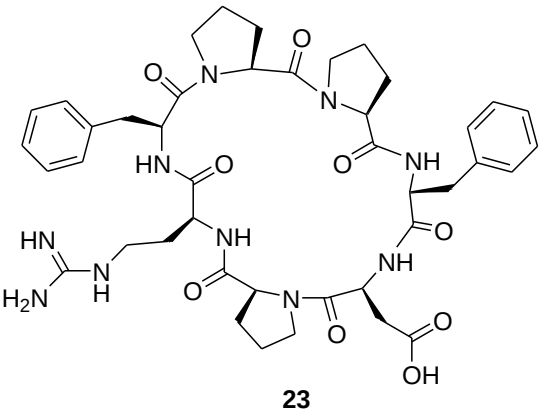

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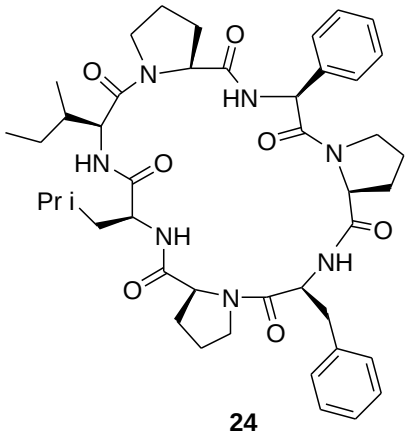

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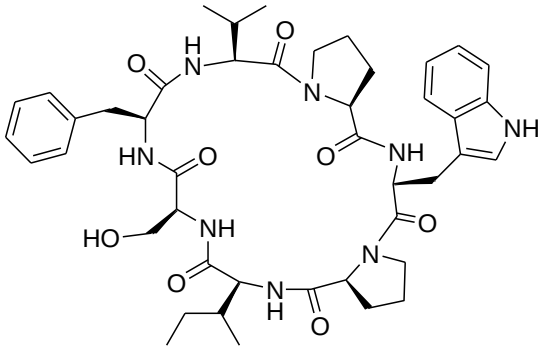

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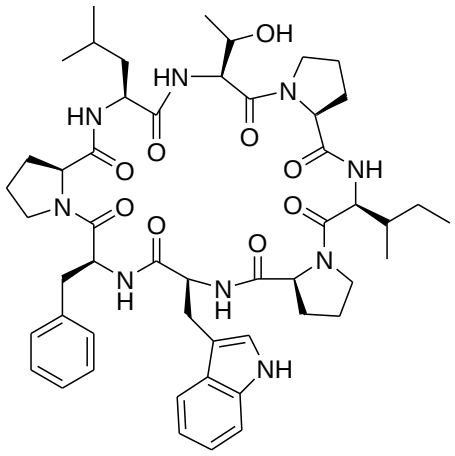

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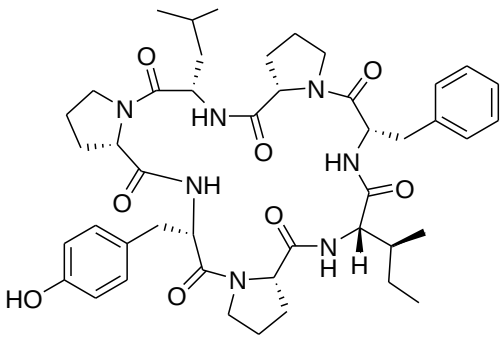

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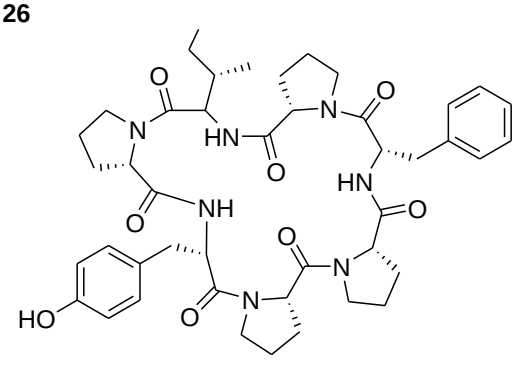

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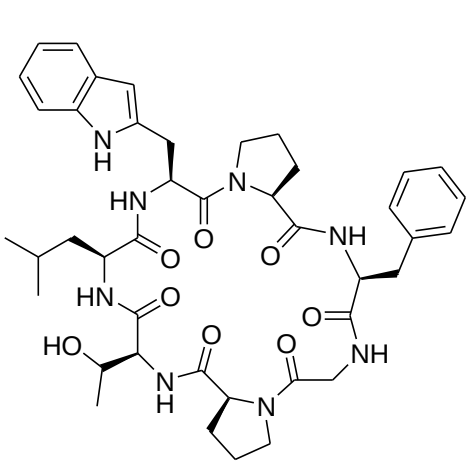

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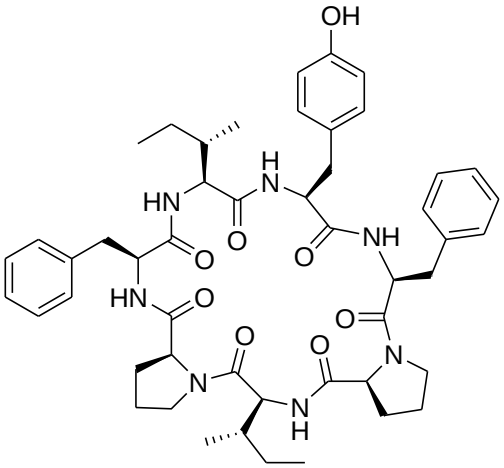

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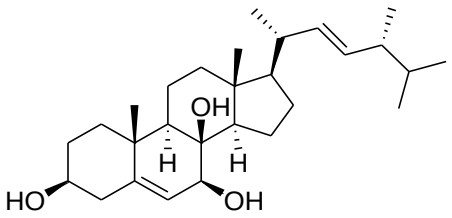
1.2.3 Miscellaneous compounds

Apart from the pyrrole carboxylic acid alkaloids and cyclic peptides described above, some other classes of compounds isolated from *Stylissa* sponges include steroids and ceramides. The examples for the sterols are stylisterols A-C (**31**-**33**), hatomasterol (**34**), from *Stylissa* sp. (Mitome et al, 2005), ergosterol peroxide (**35**), 5α,9α-epidioxy-8α,14α-epoxy-(22*E*)-ergosta-6,22-dien-3α-ol (**36**) from *Stylissa* cf. *massa* (previously *Ciocalapata* sp.) (Wattanapiromsakul et al, 2009), and that for the ceramides are STL-4 (**37**) and STL-8 (**38**) from *S. frabeliformis* (Uchimura et al, 1997).



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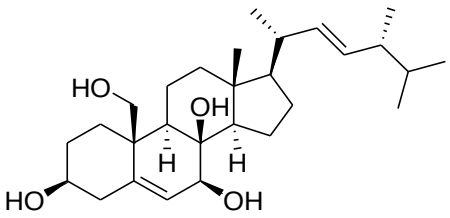

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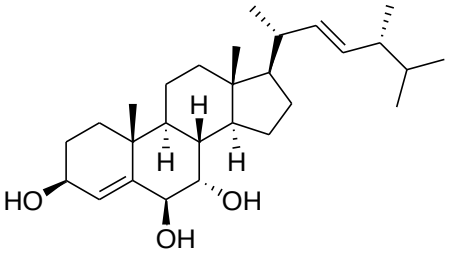

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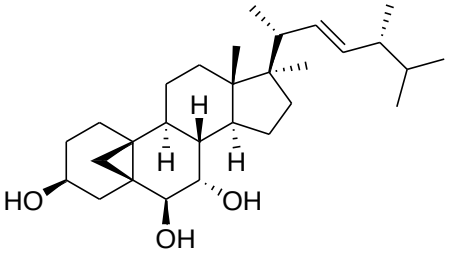

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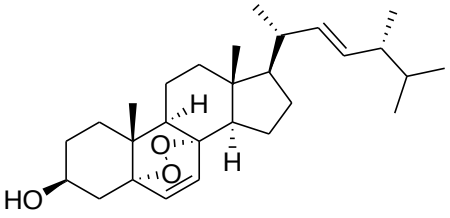

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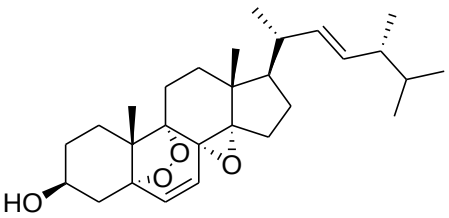

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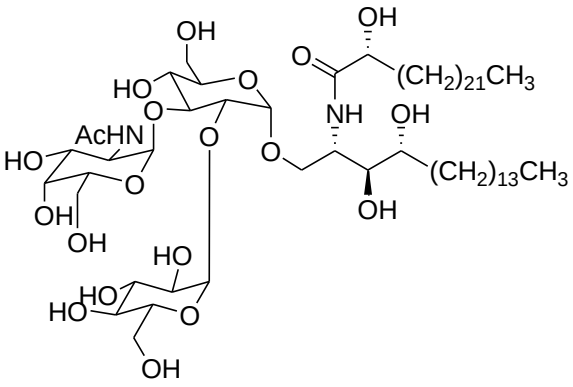

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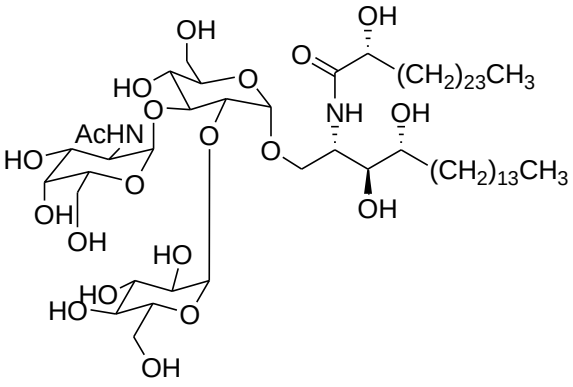

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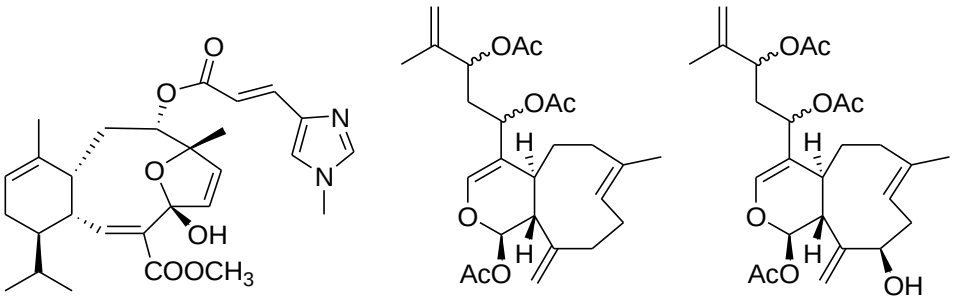
1.3 The soft coral *Eleutherobia* sp. and their chemical constituents

The soft corals of the genus *Eleutherobia* sp. belong to family Alcyoniidae, order Alcyonacea, class Anthozoa. Twenty-two species have been identified. The soft corals are found distributing in South Africa, Australia, the Philippines, Indonesia, and Japan (WoRMs, 2014).

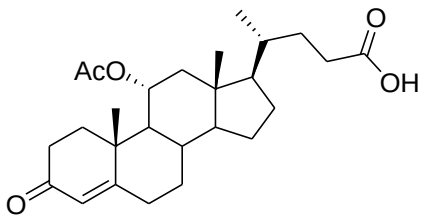
To date, there have been 16 compounds isolated from *Eleutherobia* soft corals, including *E. aurea*, *E. albiflora*, and a few unidentified specimens. The soft corals of the genus *Eleutherobia* in fact have been studied comparatively very little. Apart from the very well-known eleutherobins, the major group of compounds from *Eleutherobia* is diterpenes of eunicillin class. The example of terpenoids in this group are sacodictyin A (**39**) (Ketzinel et al, 1996), zahavins A (**40**) and B (**41**) (Rudi et al, 1995; Hooper et al, 1997), 9-deacetoxy-14,15-deepoxyxeniculin (**42**), xeniolide C (**43**) (Hooper et al, 1997) from *E. aurea* and, minabein 1 (**44**) from *Eleutherobia* sp. (Lievens et al, 2004).

Probably the most famous eunicillin diterpenes from *Eleutherobia* soft corals, eleutherocin (**45**) is a diterpene glycoside first isolated from *E. albiflora* (Lindel et al, 1997). The compound was cytotoxic with a paclitaxel-like mitotic-poison mechanism. Related diterpene glycosides from *Eleutherobia* soft coral also include eleutherosides A (**46**) and B (**47**) (Ketzinel et al, 1996).



39 **40** **41**

The other class of isolated compounds from the soft coral *Eleutherobia* spp. is steroids. These include a glycosidic cholesterol analog, auroside (**48**) (Ivanchina et al, 2011) and cholic acid derivatives; 11-acetyl-3-oxo-chol-4-en-24-oic acid (**49**), 3-oxo-chol-1,4,22-trien-24-oic acid (**50**), 3-oxo-chol-4-en-24-oic acid (**51**), 3-oxo-chol-4,22-dien-24-oic acid (**52**), and 3-oxo-chol-1,4-dien-24-oic acid (**53**) from the soft coral *Eleutherobia* sp. (Lievens et al, 2004).



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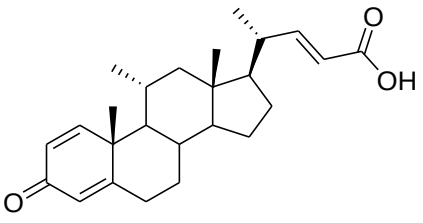

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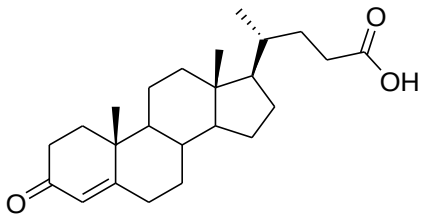

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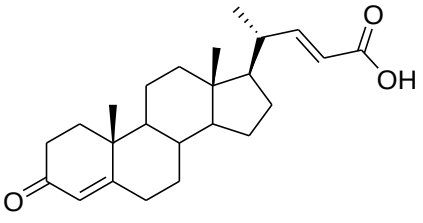

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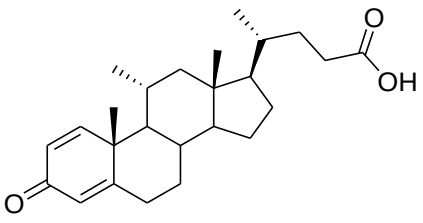

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1.4 Terpenoids isonitriles and related derivatives

Terpenoids bearing isonitrile and related functionalities, i.e., isothiocyanate, isocyanate, and formamide, are one of the exclusively marine-derived natural products. Major sources of terpenoids in this group are the haplosclerid sponges including those in genera *Amphimedon, Axinella*, *Ciocalypta*, *Cribochalina*, *Cymbastella*, *Halichondria*, *Hymeniacidon*,

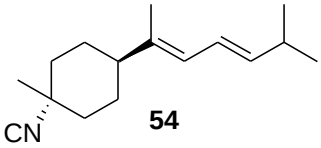
Pseudoaxinella, and *Stylissa* (Civatta et al, 2005; Avilés et al, 2013). Additional sources also include nudibranchs in genus *Phyllidia* (Fusetani et al, 1991), which feed on Halichondrid sponges, hence accumulating the compounds through their diets (Molinski et al, 1987).

Chemical structures of terpenoids in this class vary from sesquiterpenes of spiroaxane, axane, cadinane, eudesmane, aromadendrane, and pupukeanane classes, to diterpenes of amphilectane, cycloamphilectane, and kalihinane classes. As for the nitrogenated functionalities, either isonitrile, isocyanate, isothiocyanate, or formamide, have been found associated with the terpenoids in each class as stated above without any specific structural preference.

1.4.1 Sesquiterpenoids

As mentioned above, sesquiterpenoids containing isonitrile and related functional groups have been devised in a wide range of chemical skeletons, from a monocyclic sesquiterpenes as in 3-isocyanotheonellin, to dicyclic ones as in axisonitrile-3. Rearranged terpenoids toward bridged tricyclic structures have also been reported.

Theonellins are bisabolane sesquiterpene that have been found in various sponges and also in several nudibranch species. Terpenoids in this family include 3-isocyano-theonellin (**54**) [from the sponges *Halichondria* cf. *lendenfeldi* (Kassühlke et al, 1991), and *Rhaphoxya* sp. (Wright et al, 2012), and from the nudibranchs *Phyllidia pustulosa*, synonym *Phyllidiella pustulosa*; Okino et al, 1996; Manzo et al, 2004], 3-isothiocyanatotheonellin (**55**) [from the sponges *Rhaphoxya* sp. (Wright et al, 2012), *Halichondria* sp. (Sullivan and Faulkner, 1986), and *Phycopsia* sp. (Kondempidi et al, 2009)], 7α,8α-epoxide-3-isothiocyanatotheonellin (**56**) [from the sponge *Phycopsia* sp. (Kondempidi et al, 2009)], 3-isocyanatotheonellin (**57**) [from the sponge *Rhaphoxya* sp. (Wright et al, 2012)], 3-formamidotheonellin (**58**) [from the sponges *Halichondria* sp. (Sullivan and Faulkner, 1986), *Phycopsia* sp. (Kondempidi et al, 2009), *Rhaphoxya* sp. (Wright et al, 2012), and *Axinyssa* sp. (Li et al, 1999)].



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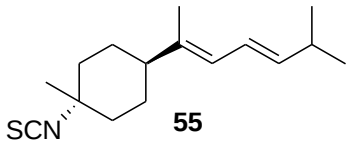

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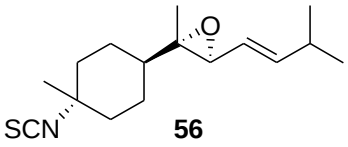

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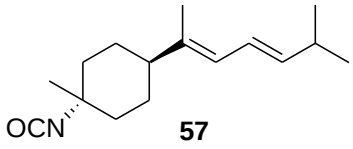

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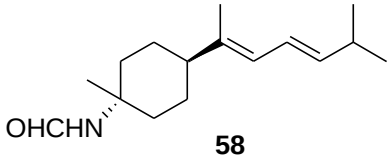

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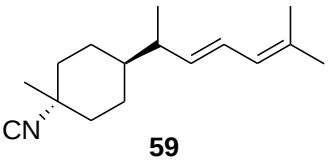

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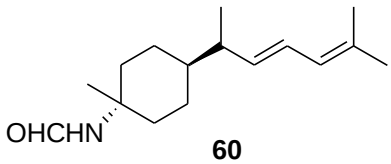
Apart from theonellins, several other bisabolane-derived isonitrile and derivatives have also been reported. This include 3-isocyanobisabolane-8,10-diene (**59**) [from the nudibranch *Phyllidia pustolosa* (Kassühlke et al, 1991)], 3-formamidobisabolane-8,10-diene (**60**) [from the sponge *Halichondria* cf. *lendenfeldi* and the nudibranch *Phyllidia pustolosa* (Kassühlke et al, 1991)], 3(*E)*-isocyanobisabolane-7,10-diene (**61**) [from the sponge *Axinyssa* sp. (Garson and Simpson, 2004)], 3-formamidobisabolane-14(7),9-diene (**62**) and 3-formamido-bisabolane-14(7),9-diene-8-ol (**63**) [from the sponge *Axinyssa* sp. (Li et al, 1999)], 3-isocyano-bisabolane-14(7),10-diene (**64**) [from the nudibranch *Phyllidiella pustulosa* (Manzo et al, 2004)], 3-formamido-8-methoxybisabolane-9-en-10-ol (**65**) [from the sponge *Axinyssa* sp. (Li et al, 1999)], 7(*R*)-isocyanobisabolane-10-ene (**66**) and 7(*R*)-isocyanatobisabolane-10-ene (**67**) [from the sponge *Ciocalypta* sp. (Gulavita et al, 1986)], Δ^2-7-isothiocyanato-7,8-dihydro-α-bisabolene (**68**) [from the sponge *Halichondria* sp. (Sullivan and Faulkner, 1986)], and Δ^3-7-isothio-cyanato-7,8-dihydro-α-bisabolane (**69**) [from the sponge *Rhaphoxya* sp. (Wright et al, 2012)].



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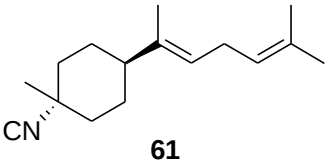

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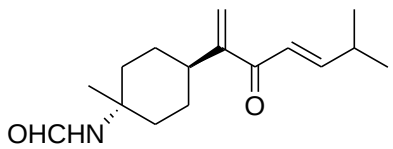

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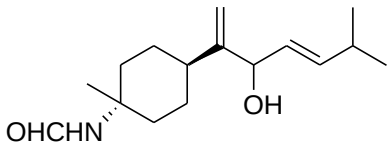

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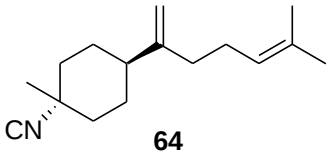

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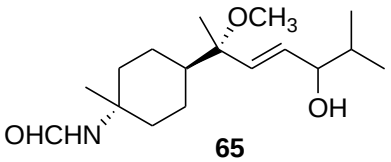

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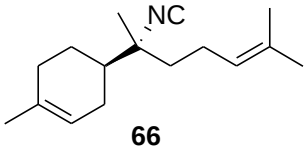

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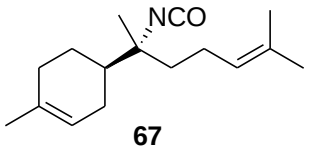

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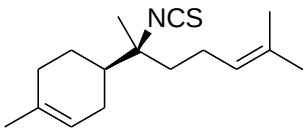

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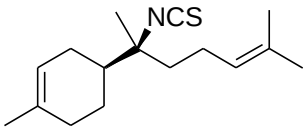


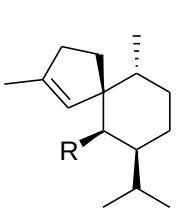
69

Among all bisabolane derivatives discussed above, only **54** and **69** have been reported to have biological activities. Compound **54** showed a potent antifouling activity against *Balanus amphitrite* lavae (IC_{50} = 0.13 µg/mL; Okino et al, 1996a), and compound **69** was toxic to brine shrimp (IC_{50} = 0.1 µg/mL; Garson and Simpson, 2004).

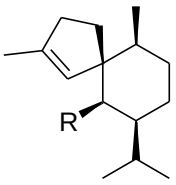
Spiroaxanes and axanes are two other classes of sesquiterpenes that are unique to marine natural products. Axisonitrile-3 (**70**) was reported from the sponges *Acenthella klethra* (Angerhofer and Pezzuto, 1992), *Ac. cavernosa* (Fusetani et al, 1992; Clark et al, 2000), and *Acanthella* sp. (Yan et al, 2006), and from the nudibranch *Phyllidia pustulosa* (Okino et al, 1996a). The compound showed the potent antimalarial activity against *Plasmodium falciparum* D6 and W2 (IC_{50} = 142.0 and 16.5 ng/mL, respectively) with undetectable cytotoxicity to KB-3 cells at 20 µg/mL (Angerhofer and Pezzuto, 1992). Compound **70** also showed antifouling activity against barnacle *Balanus amphitrite* larvae (IC_{50} = 3.2 µg/mL; Okino et al, 1996a).

Several other spiroaxanes have been isolated from various marine organisms. These include axisothiocyanate-3 (**71**) [from the sponges *Acanthella klethra* (Angerhofer and Pezzuto, 1992) and *Ac. cavernosa* (Hirota et al, 1996; Clark et al, 2000)], 10-*epi*-axisonitrile-3 (**72**) [from the sponge *Geodia exigua* (Uy et al, 2003), and the nudibranch *Phyllidia pustulosa* (Okino et al, 1996a)], (+)-axamide-3 (**73**) [from the sponge *Ac. cavernosa* (Hirota et al, 1996)], exiguamide (**74**) [from the sponge *G. exigua* (Uy et al, 2003)], 3-oxo-axisonitrile-3 (**75**) [from the sponge *Acanthella* sp. (Yan et al, 2006)], (-)-axisonitrile-3 (**76**) [from the sponges *Ac. cavernosa* (Hirota et al, 1996), and *G. exigua* (Uy et al, 2003)], (1*R**,2*S**,10*S**)-1-isocyano-6-axene (**77**) and (1*R**,2*S**,10*S**)-1-isothiocyanato-6-axene (**78**) [from the sponge *Ac. acuta* (Mayol et al, 1987)], (2*R**,5*R**,10*R**)-2-isothiocyanato-6-axene (**79**), and (2*R**,5*R**,10*R**)-2-formamido-6-axene (**80**) [from the sponge *Trachyopsis aplysinoides* (He and Faulkner, 1989)].

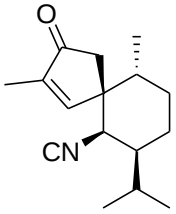
Several spiroaxane analogs also exhibited the interesting biological activities. For examples, compound **76** was cytotoxic toward HepG2 cell line (IC_{50} = 1.3 μM; Prawat et al, 2011), and compound **74** has been reported to inhibit the embryogenesis of sea urchin *Hemicentrotus pulcherrimus* (IC_{50} = 0.4 - 12.0 μM; Uy et al, 2002).



70 R= NC
71 R= NCS
73 R= NHCHO



72 R= NC
74 R= NHCHO



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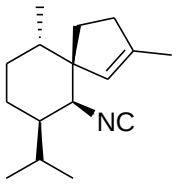
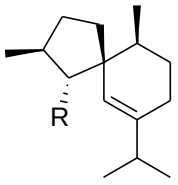
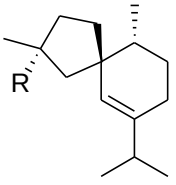


76



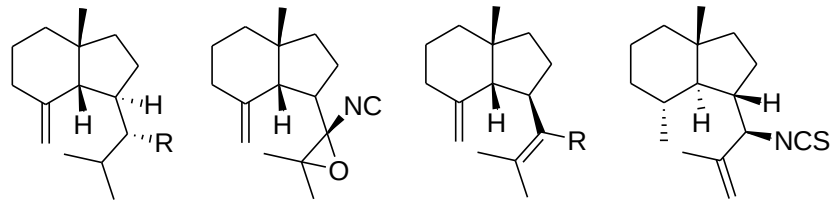
77 R= NC
78 R= NCS



79 R= NCS
80 R= NHCHO

Seven axane-type sesquiterpenes associated with isonitrile and related functional groups have been reported. These include axisonitrile 1 (**81**) [from the sponges *Axinella cannabina* (Fattorusso et al, 1974; Fattorusso et al, 1975), *Acanthella cannabina*, and the nudibranch *Phyllidia pulitzeri* (Kassühlke et al, 1991)], axisothiocyanate 1 (**82**), and axamide 1 (**83**) [from the sponge *Ax. cannabina* (Fattorusso et al, 1974; Fattorusso et al, 1975)], caverno-isonitrile (**84**) [from the sponge *Ac. carvernosa* (Fusetani et al, 1992)], (-)-axisonitrile 4 (**85**), and (+)-axamide 4 (**86**) [from the sponge *Ax. cannabina* (Ohkubo et al, 1995)], and 10-isothio-cyanato-11-axene (**87**) [from the sponge *Ac. cavernosa* (Hirota et al, 1996)].

The majority of susquiterpenes with isonitrile and related functionalities falls into three groups, cadinane, eudesmane, and aromadendrane. Similar to other cadinane and eudesmane sesquiterpenes readily described, the core terpenoid skeletons of the cadinanes and eudesmanes are very well reserved, with almost consistent substitution positions at C-4, C-7, and C-10 for cadinanes and at C-1, C-5, and C-8 for eudesmannes, and the variation took place largely at the nitrogenated functional groups themselves.



81 R= NC
82 R=NCS
83 R=NHCHO

84

85 R= NC
86 R= NHCHO

87

Marine-derived cardinanes isolated to date include 10α-isocyano-4-amorphene (**88**) [from the sponges *Halichondria* sp. (Bureson et al, 1975), *Acanthella* cf. *carvernosa* and the nudibranch *Phyllidia ocellata* (Fusetani et al, 1992)], 10α-isothiocyanato-4-amorphene (**89**) [from the sponges *Halichondria* sp. (Bureson et al, 1975), *Axinyssa* sp. (Marcus et al, 1989; Zubia et al, 2008), *Acanthella* sp. (Yan et al, 2006), *Ac. carvernosa* (Clark et al, 2000), *Stylissa*

sp. (Mitome et al, 2004) and *Axinyssa aplysinoides* (Sork et al, 2008)], (1*R**,6*R**,7*S**,10*S**)-10-isothiocyanato-4-cadinene (**90**) and (1*S**,2S*,5S*,6S*,7*R**, 8*S**)-13-isothiocyanatocubebane (**91**) [from the sponge *Stylissa* sp. (Mitome et al, 2004)], 10α-formamido-4-amorphene (**92**) [from the sponge *Halichondria* sp. (Burreson et al, 1975)], and 10-isothiocyanato-4,6-amorphene (**93**) [from the sponges *Ax. aplysinoides* and *Ax. fenestratus* (Sork et al, 2008)].

Halipanicine (**94**) was isolated from the sponges *Ax. aplysinoides* and *Halichondria panacea* (Compagnone and Faulkner, 1995). (3*S**,5*R**,6*R**,9*R**)-3-Isocyano-1(10) -cadinene (**95**) and (3*S**,5*R**,6*R**,9*R**)-3-formamido-1(10)-cadinene (**96**) were isolated from the sponge *Ax. aplysinoides* (Compagnone and Faulkner, 1995).

Epipolasin A (**97**) was isolated from the sponges *Acanthella* sp. (Burgoyne et al, 1993; Yan et al, 2006) and *Axinyssa* sp. (Kodama et al, 2003). Its isonitrile (**98**) and formamide (**99**) derivatives were isolated from the sponge *Acanthella* sp., and the nudibranch *Cadlina luteomarginata* (Burgoyne et al, 1993). 4α-Isocyanogorgen-11-ene (**100**) was isolated from the nudibranch *Phyllidia varicosa* and *P. pustulosa*. 4α-Isothiocyanatogorgen-11-ene (**101**) was isolated from the nudibranch *Phyllidia varicosa*, and 4α-formamidogorgen-11-ene (**102**) was from the nudibranch *P. pustulosa* (Kassühlke et al, 1991).

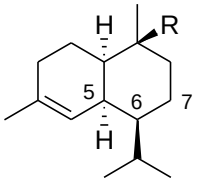
(1*S**,4*S**,7*R**,10*S**)-10-Isocyano-5-cadinen-4-ol (**103**) was isolated from the nudibranch *Phyllidia pustulosa*. **103** exhibited a good antifouling activity against barnacle *Balanus amphitrite* larvae (IC_{50}= 0.17 μg/mL; Miyaoka et al, 1998). Axiplyns C (**104**) and E (**105**) were found in the sponge *Axinyssa aplysinoides*, along with axiplyns A (**106**), B (**107**), and D (**108**). Compounds **104**, **106**, and **107** were lethal to *Artemia salina* (IC_{50} = 1.8, 1.6, and 1.5 μg/mL, respectively; Sork et al, 2008).

The eudesmane sesquiterpenes containing isonitrile and related functionalities include, 11-isocyano-7β*H*-eudesm-5-ene (**109**) from the sponges *Axinella cannabina* (Ciminiello et al, 1987a), *Acanthella* sp. and the nudibranch *Cadlina luteomarginata* (Burgoyne et al, 1993). 11-Isothiocyanato-7β*H*-eudesm-5-ene (**110**) was isolated from the sponges *Acanthella* sp.

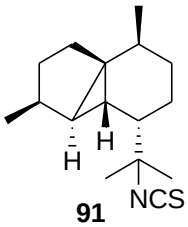
(Kassühlke et al, 1991), *Ax. cannabina* (Ciminiello et al, 1987a), *Ac. klethra* (Angerhofer and Pezzuto, 1992) and the nudibranch *C. luteomarginata* (Burgoyne et al, 1993). Compound **110** showed a weak antiplasmodial activity against *Plasmodium falciparum* D6 and W2 (IC_{50} = 2.24 and 0.61 µg/mL, respectively; Angerhofer and Pezzuto, 1992). 11-Formamido-7β*H*-eudesm-5-ene (**111**) was isolated from sponge *Ax. cannabina* (Ciminiello et al, 1987a).

ent-Stylotelline (**112**) was isolated from the sponge *Stylotella* sp. (Pais et al, 1987) and the nudibranch *Phyllidia pustulosa* (Manzo et al, 2004). Acanthellin 1 (**113**) was from the sponges *Acanthella acuta* and *Axinella cannabina*, together with its isothiocyanate (**114**) and formamide (**115**) analogs (Ciminiello et al, 1984). Acanthine B (**116**) was reported from the sponges *Ac. acuta* and *Ax. Cannabina*, along with its isothiocyanate derivative (**117**) (Ciminiello et al, 1987b ; Mayol et al, 1987). The formamide derivative (**118**) of **116** was reported from the sponge *Ax. cannabina* (Ciminiello et al, 1987b) and the nudibranch *Cadlina luteomarginata* (Burgoyne et al, 1993).

Halichondrin C (**119**) was isolated from the sponge *Halichondria* sp. (Ishiyama et al, 2005), and its Cu(I) complex (**120**) was reported from the sponge *Halichondria* sp. (Ishiyama et al, 2008). (1*R*,5*R*,6*R*,8*R*)-Dec[4.4.0]ane-1,5-dimethyl-8-(1-methylethenyl)-5-iso-thiocyanate (**121**) was isolated from the sponges *Acanthella* sp., *Ac. klethra* (Angerhofer and Pezzuto, 1992) and the nudibranch *C. luteomarginata* (Burgoyne et al, 1993). The 8-epimer of **121**, compound **122** was isolated from the sponge *Ac. klethra,* and showed a weak antiplasmodial activity against *Plasmodium falciparum* D6 and W2 (IC_{50}= 4.0 and 0.55 µg/mL, respectively; Angerhofer and Pezzuto, 1992; König et al, 1992).



88 R= NC
89 R= NCS, 5Hα
90 R= NCS, 5Hβ
92 R= NHCHO
93 R= NCS, $\Delta^{6,7}$



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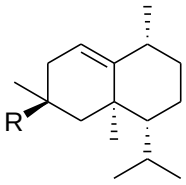
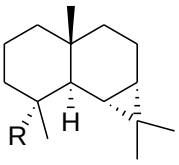
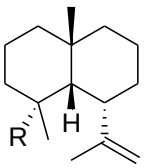


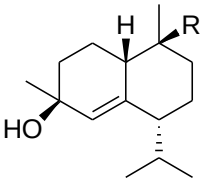
94 R= NCS
95 R= NC
96 R= NHCHO



97 R= NCS
98 R= NC
99 R= NHCHO



100 R=NC
101 R= NCS
102 R= NHCHO



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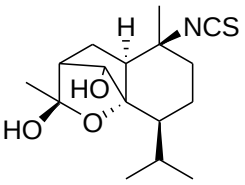
104 R= NCS



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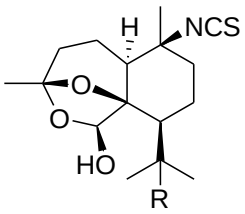
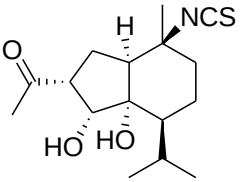


106 R= H
107 R= OH



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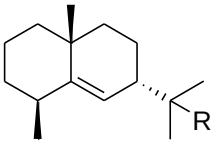
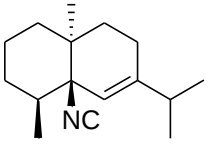


109 R= NC
110 R= NCS
111 R= NHCHO



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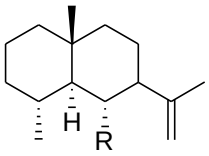
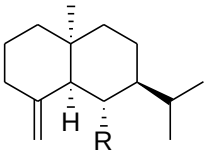
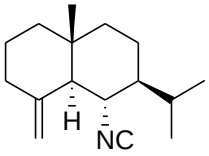


113 R= NC
114 R= NCS
115 R=NHCHO



116 R= NC
117 R= NCS
118 R= NHCHO



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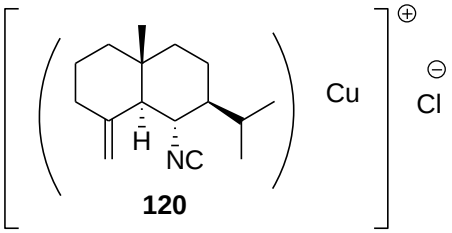

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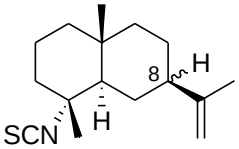


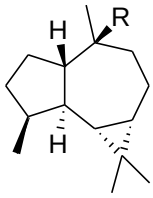
121 8-Hα
122 8-Hβ

Another class of bicyclic sesquiterpene with isonitrile and related functionalities is aromadendranes. The compounds in this class include axisonitrile 2 (**123**), axisothiocyanate 2

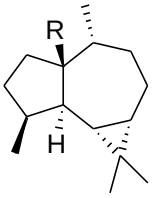
(**124**), and axamide 2 (**125**), all of which were isolated from the sponge *Axinella cannabina* (Fattorusso et al, 1974; Fattorusso et al, 1975).

In addition, an isomer of **125**, compounds **126** and **127** were found in the sponges *Axinyssa* sp. (Kodama et al, 2003), *Halichondria* sp. (Prawat et al, 2011) and the nudibranch *Hexabranchus sanguineus* (Zhang et al, 2007). (+)-10(R)-Isothiocyanatoalloaroma-dendrane (**128**) was found in the sponges *Acanthella cavernosa* (Hirota et al, 1996), and *Acanthella* sp. (Yan et al, 2006), and it 10-epimer (**129**) was also isolated from the sponge *Acanthella* sp. (Yan et al, 2006).

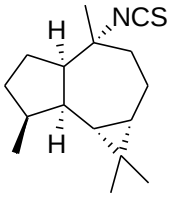
A series of isonitrile, isothiocyanate, and formamide aromadendranes, **130** - **132** were isolated from the sponge *Ax. cannabina* (Ciminiello et al, 1987a) and a 10-epimer (**133**) of **131** was isolated from the sponge *Acanthella* sp. (Yan et al, 2006). (1*S**,4*S**, 5*R**,10*S**)-10-Isothiocyanatoguaia-6-ene (**134**) was isolated from the sponge *Trachyopsis aplysinoides* (He and Faulkner, 1989) and its isonitrile derivative (**135**) was isolated from the nudibranch *Phyllidia pustulosa* (Manzo et al, 2004). The isonitrile and isothiocyanate aromadentranes **136** - **139** were isolated from the sponge *Ac. acuta* (Mayol et al, 1987).



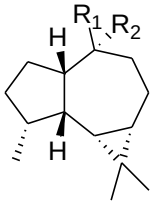
123 R= NC
124 R= NCS
125 R= NHCHO



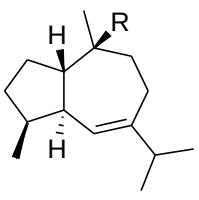
126 R= NCS
127 R= NHCHO



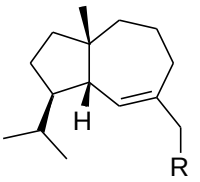
128 α–NCS
129 β–NCS



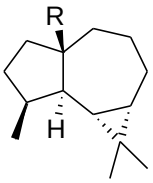
130 R_1= Me, R_2= NC
131 R_1= Me, R_2= NCS
132 R_1= Me, R_2= NHCHO
133 R_1= NCS, R_2= Me



134 R= NCS
135 R= NC

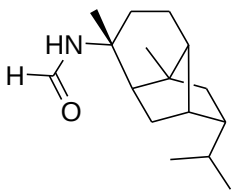


136 R= NC
137 R= NCS



138 R= NC
139 R= NCS

Pupukeananes, bearing a bridged tricyclic skeleton, are among the most complicated sesquiterpenes in this series. The compounds in this class include abeopupukeanane (**140**) from the nudibranch *Phyllidia coelestis* (Jaisamut et al, 2013). 9-Isocyanopupukeanane (**141**) was isolated from the sponges *Ciocalypta* sp. (He and Faulkner, 1989), and *Axinyssa* sp. (Marcus et al, 1989), and from the nudibranchs, *P. bourguini* and *P. pustulosa* (Kassühlke et al, 1991). The 9-epimer (**142**) of **141** was isolated from the nudibranchs *P. bourguini* (He and Faulkner, 1989; Kassühlke et al, 1991) and *P. pustulosa* (Kassühlke et al, 1991). 2-Isocyano-pupukeanane (**143**) was reported from the sponges *Ciocalypta* sp. (He and Faulkner, 1989; Kassühlke et al, 1991), *Axinyssa* sp. (Marcus et al, 1989), and from the nudibranch *P. bourguini* (He and Faulkner, 1989; Kassühlke et al, 1991). 2-Isothiocyanatopupukeanane (**144**) and 2-formamidopupukeanane (**145**) were reported from the sponge *Axinyssa* sp. (Marcus et al, 1989). 5-Isothiocyanatopupukeanane (**146**) was isolated from the sponge *Axinyssa* sp. (Marcus et al, 1989). 2-Isothiocyanatotrachyopsane (**147**) was found in the sponge *Ax. aplysinoides* (formerly *Trachyopsis*) (He et al, 1992) and the nudibranch *P. varicosa*. Allopupukeanane (**148**) was found in the nudibranch *P. pustulosa* (Kassühlke et al, 1991).



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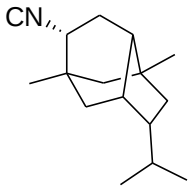

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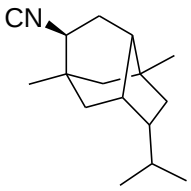

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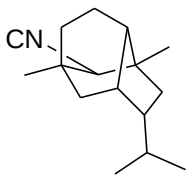

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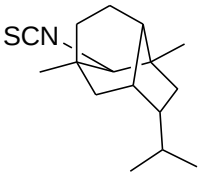

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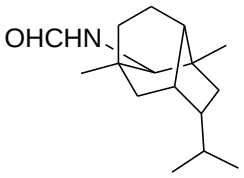

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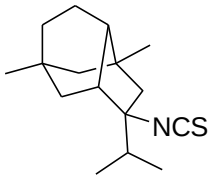

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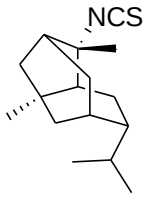

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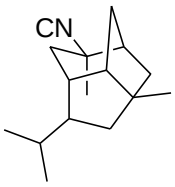


148

1.4.2 Diterpenoids

Diterpenes bearing isonitrile and related functionalities from marine natural products are classified into the groups of amphilectane, cycloamphilectane, and kalihinane. For the amphilectane diterpenes, these include (1*S**,3*S**,4*R**,7*S**,8*R**,12*S**,13*S**)-7,15-diisocyano-amphilecta-11(20)-ene (**149**) from the sponges *Cribochalina* sp. (Ciavatta et al, 1999; Ciavatta et al, 2005), *Svenzea flava* (Avilés et al, 2013), and *Pseudoaxinella flava* (Lamoral-Theys et al, 2011). The compound showed cytotoxicity against U373, Hs683, A549 NSCLC, LoVo, and SKMEL-28 cell lines (IC_{50}= 10.0, 4.0, 16.0, 3.0, and 32.0 µM, respectively; Lamoral-Theys et al, 2011). (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-8,15-Diisocyanoamphilect-11(20)-ene (**150**) was isolated from the sponges *Hymeniacidon amphilecta* (Wratten and Faulkner, 1978), *S. flava* (Avilés et al, 2013), *P. flava* (Lamoral-Theys et al, 2011), *Cribochalina* sp. (Ciavatta et al, 1999; 2005), *Hymeniacidon* sp. (Avilés and Rodríguez, 2010), and the nudibranch *Phyllidiella pustolosa* (Manzo et al, 2004). Compound **150** showed antimalarial activity against *Plasmodium falciparum* K1 (IC_{50} = 0.09 µM; Wattanapiromsakul et al, 2009), cytotoxicity against U373, Hs683, A549 NSCLC, LoVo, and SKMEL-28 cell lines (IC_{50} = 25.0, 50.0, 42.0, 3.0, 6.0 µM, respectively; Lamoral-Theys et al, 2011), and showed antitubercular against *Mycobacterium tuberculosis* H_{37}Rv (MIC = 3.2 µg/mL; Avilés and Rodríguez, 2010).

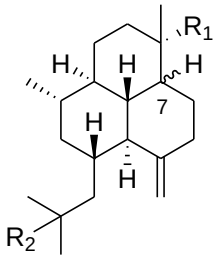
(1*S**,3*S**,4*R**,7*S**,8*R**,12*S**,13*S**)-7-Isocyano-15-isothiocyanatoamphilecta-11-(20)-ene (**151**) was isolated from the sponge *Cribochalina* sp. (Ciavatta et al, 1999; Ciavatta et al, 2005) and (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-7-isocyano-15-isothiocyanatoamphilect-11(20)-ene (**152**) was from the sponge *Cymbastela hooperi* (Wright and König, 1996). (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-8-Isocyano-15-formamidoamphilecta-11(20)-ene (**153**) was isolated from the sponges *Hymeniacidon amphilecta* (Wratten and Faulkner, 1978), and *Svenzea flava* (Avilés et al, 2013).

Amphilectene diterpenes containing isonitrile are (1*R**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-7-isocyanoamphilecta-10,14-diene (**154**) from the sponges *Cymbastela hooperi* (Wright and König, 1996) and *Stylissa* sp. (Mitome et al, 2004). Compound **156** exhibited cytotoxicity against HeLa cells (IC_{50}= 20.0 μM; Mitome et al, 2004). (1*R**,3*S**,4*R**,7*S**,8*S**,13*R**)-7-Isocyanoamphilecta-11,14-diene (**155**) was isolated from the sponge *C. hooperi* (Wright and König, 1996), and showed antiplasmodial activity against *Plasmodium falciparum* D6 and W2 (IC_{50} = 9.3 and 25.6 ng/mL, respectively; Wright and König, 1996).

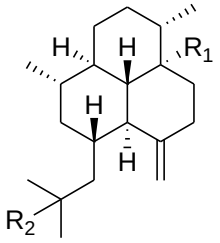
(1*R**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-7-Isocyanoamphilecta-11(20),14-diene (**156**) was reported from the sponges *Adocidae* sp. (Sharma et al, 1992), *Cymbastela hooperi* (Wright and König, 1996), and *Pseudoaxinella flava* (Lamoral-Theys et al, 2011). The compound showed an antiplasmodial activity against *P. falciparum* D6 and W2 (IC_{50} = 14.1 ng/mL for both D6 and W2; Wright and König, 1996). (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-7-Isocyanoamphilecta-11(20),15-diene (**157**) was isolated from the sponges *C. hooperi* (Wright and König, 1996), *Stylissa* cf. *massa* (formerly *Ciocalapata* sp.) (Wattanapiromsakul et al, 2009), and *P. flava* (Lamoral-Theys et al, 2011). Compound **157** showed an antiplasmodial activity against *P. falciparum* K1 (IC_{50} = 1.07 μM; Wattanapiromsakul et al, 2009). (1*S**,3*S**,4*R**,7*S**,8*R**,12*S**,13*S**)-7-Isocyanoamphilecta-11(20),15-diene (**158**) was isolated from the sponge *Cribochalina* sp. (Ciavatta et al, 1999; Ciavatta et al 2005). (1*S**,3*S**,4*R**,7*S**,8*R**,12*S**,13*R**)-8-Isocyano-

amphilecta-10,14-diene (**159**) was isolated from the sponge *Halichondria* sp., and showed an antibacterial activity against *S. aureus* and *B. subtilis* (Mayol et al, 1987).

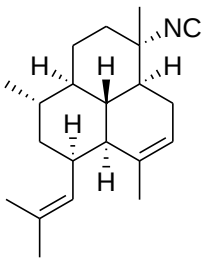
(3*S**,4*R**,7*S**,8*S**,11*S**,13*S**)-8-Isocyanoamphilecta-1(12),14-diene (**160**) was isolated from the sponge *Stylissa* sp, and showed a cytotoxicity against HeLa cells (IC_{50}= 11.2 μM; Mitome et al, 2005). (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-8-Isocyano-amphilecta-11(20),14-diene (**161**) was isolated from the sponges *Stylissa* sp. (Mitome et al, 2005) and *Stylissa* cf. *massa* (formerly *Ciocalapata* sp.), and exhibited antiplasmodial activity against *Plasmodium falciparum* K1 (IC_{50} = 0.44 μM; Wattanapiromsakul et al, 2009). (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**, 13*S**)-8-Isocyanoamphilect-11(20),15-diene (**162**) was isolated from sponge *S. massa* (formerly *Ciocalapata* sp.), and showed antiplasmodial activity against *P. falciparum* K1 with IC_{50}= 0.98 μM (Wattanapiromsakul et al, 2009).



149 R_1= NC, R_2= NC, 7βH
151 R_1= NC, R_2= NCS, 7βH
152 R_1= NC, R_2= NCS, 7αH



150 R_1= NC, R_2= NC
153 R_1= NC, R_2= NHCHO



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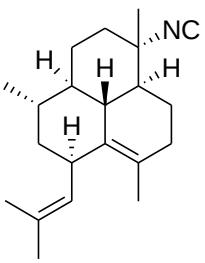

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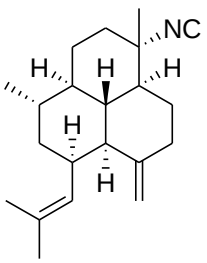

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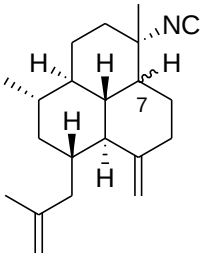
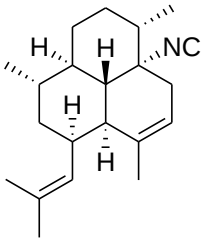


157 7αH
158 7βH



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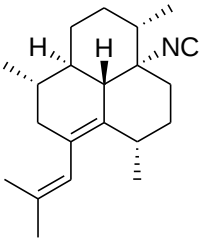

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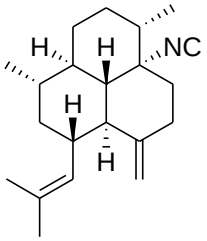

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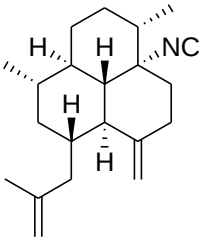
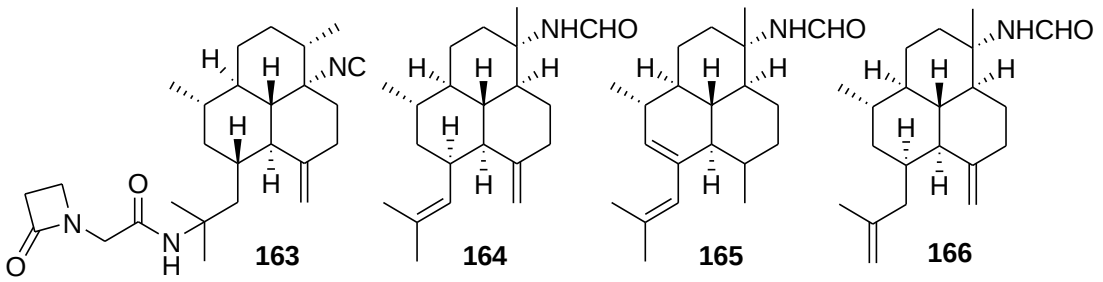
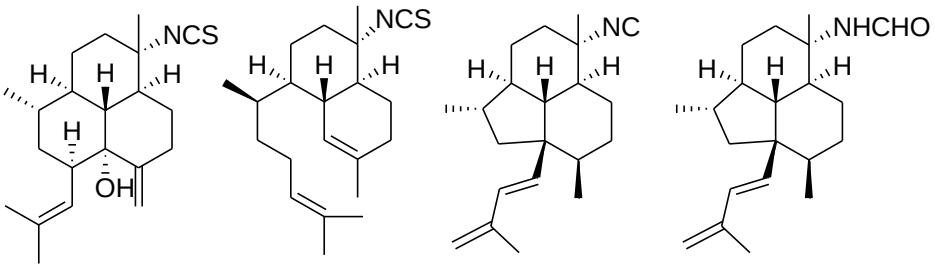


162

Monamphilectine A (**163**) was isolated from the sponge *Hymeniacidon* sp., and showed antiplasmodial activity against *Plasmodium falciparum* W2 (IC_{50} = 0.60 μM) and antitubercular activities against *Mycobacterium tuberculosis* H_{37}Rv (MIC = 15.3 μg/mL; Avilés and Rodríguez, 2010). (1*R**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-7-Formamidoamphilecta-11(20),14-diene (**164**) and (1*R**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-7-formamidoamphilecta-1,14-diene (**165**) were isolated from the sponge *Axinella* sp. Both compounds showed nitric oxide production reducing properties (IC_{50} = 0.1 to 4.3 μM; Lucas et al, 2003). (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-7-Formamidoamphilecta-11(20),15-diene (**166**) was isolated from the sponge *Cymbastela hooperi* (Wright and Lang-Unnasch, 2009). (1*R**,3*S**,4*R**,7*S**,8*S**,12*R**,13*R**)-12-hydroxy-7-Isothiocyanatoamphilecta-11(20),14-diene (**167**), (1*S**,6*R**,7*R**,10*S**,11*R**)-10-isothiocyanato-biflora-1,14-diene (**168**), and (1(14)-*E*-3*S**,4*R**,7*S**,8*S**,11*R**,12*R**,13*R**)-7-isocyanoneo-amphilecta-1(14), 15-diene (**169**), were isolated from the sponge *C. hooperi*, and showed anti-plasmodial activity against *Plasmodium falciparum* D6 and W2 (IC_{50} = 90 - 800 ng/mL; Wright and König, 1996). 7-Formamidoisoneoamphilecta-1(14),15-diene (**170**) was isolated from *Svenzea flava* (Avilés et al, 2013).

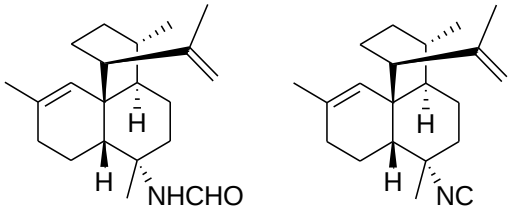


163 **164** **165** **166**



167 **168** **169** **170**

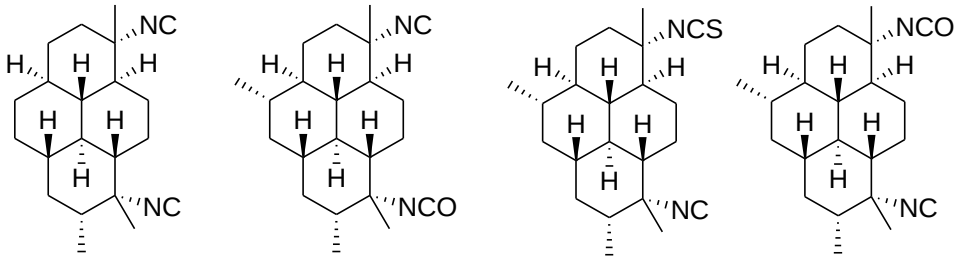
Two unprecedented neoamphilectanes have been isolated. These include (3*S**, 4*R**,7*S**,8*R**,13*R**,14*R**)-7-isocyanoneoamphilecta-11,15-diene (**171**) from the sponge *Axinella* sp. (Lucas et al, 2003) and (3*S**,4*R**,7*S**,8*R**,13*R**,14*R**)-7-isocyanoneoamphilecta-11, 15-diene (**172**) from the sponges *Adocidae* sp. (Sharma et al, 1992) and *Svenzea flava* (Avilés et al, 2013).



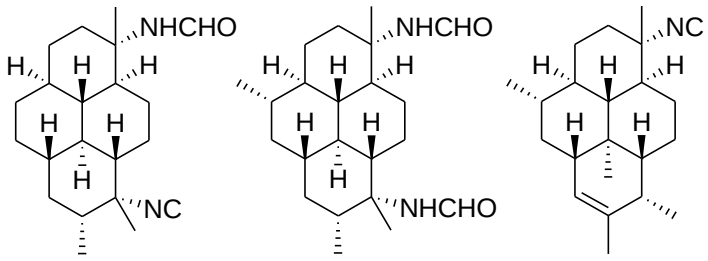
171 **172**

Additional cyclization on the amphilectane skeleton leads to the structures of cycloamphilectane. The compounds in this group include (1*S*,3*S*,4*R*,7*S*,8*S*,11*S*,12*S*,13*S*,15*R*, 20*R*)-7,20-diisocyanoisocycloamphilectane (diisocyanoadociane) (**173**) from the sponges *C. hooperi* (Wright and König, 1996), *Amphimedon* sp. (Fairweather and Mander, 2006), and showed antiplasmodial activity against *P. falciparum* D6 and W2 (IC_{50} = 4.7 ng/mL and 4.3

ng/mL, respectively; Wright and König, 1996; Wright and Lang-Unnasch, 2009). (1*S*,3*S*,4*R*,7*S*, 8*S*,11*S*,12*S*,13*S*,15*R*,20*R*)-20-Isocyano-7-isocyanatoisocycloamphilectane (**174**) was from the sponge *Cymbastela. hooperi* and, showed an antiplasmodial activity against *P. falciparum* D6 and W2 (IC_{50} = 3.2 ng/mL and 2.5 ng/mL, respectively; Wright and König, 1996). (1*S*, 3*S*,4*R*,7*S*,8*S*,11*S*,12*S*,13*S*,15*R*,20*R*)-7-Isocyanato-20-isocyanoisocycloamphilectane (**175**), (1*S*, 3*S*,4*R*,7*S*,8*S*,11*S*,12*S*,13*S*,15*R*,20*R*)-7-isothiocyanato-20-isocyanoisocycloamphilectane (**176**), (1*S*,3*S*,4*R*,7*S*,8*S*,11*S*,12*S*,13*S*,15*R*,20*R*)-7-formamido-20-isocyanoisocycloamphilectane (**177**), (1*S*,3*S*,4*R*,7*S*,8*S*,11*S*,12*S*,13*S*,15*R*,20*R*)-7,20-diformamidoisocycloamphilectane (**178**), and (1*S*,3*S*,4*R*,7*S*,8*S*,11*R*,12*R*,13*S*,20*S*)-7-isocyanoisocycloamphilect-11-ene (**179**) were isolated from the sponge *Cymbastela hooperi* (Wright and Lang-Unnasch, 2009).



173 **174** **175** **176**

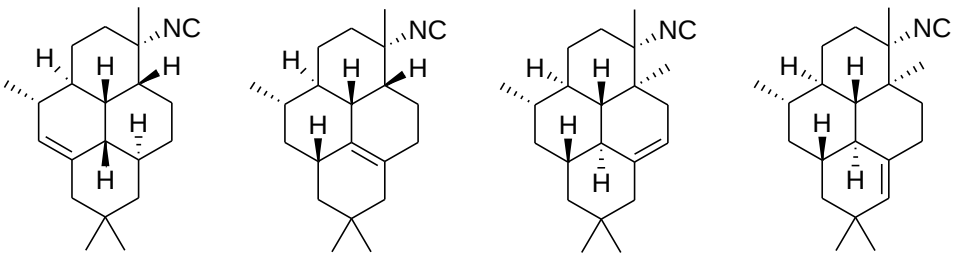


177 **178** **179**

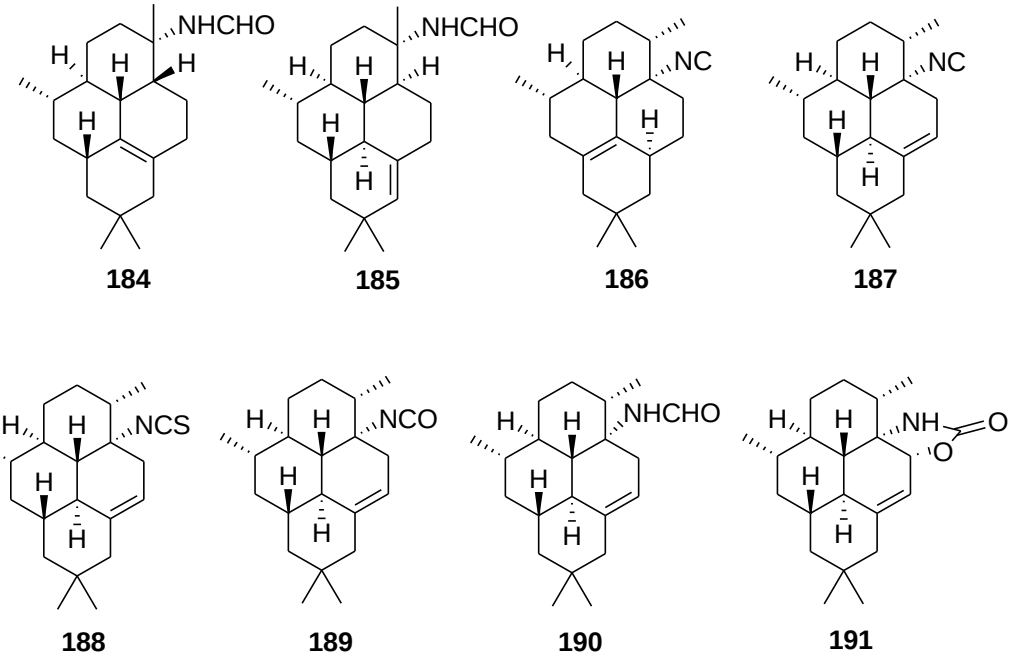
(3*S**,4*R**,7*S**,8*R**,11*S**,12*R**,13*S**)-7-isocyanocycloamphilect-1-ene (**180**) and (1*S**,3*S**,4*R**,7*S**,8*R**,13*R**)-7-isocyanocycloamphilect-11-ene (**181**) were isolated from the sponge *Halichondria* sp. (Mulinski et al, 1987). (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-7-Isocyano-

cycloamphilect-10-ene (**182**), and (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**, 13*S**)-7-isocyanocycloamphilect-11(20)-ene (**183**) were isolated from the sponge *Cymbastela hooperi* (Wright and König, 1996). (1*S**,3*S**,4*R**,7*S**,8*R**,13*R**)-7-Formamidocycloamphilect-11-ene (**184**) from sponge *Axinella* sp. (Ciasullo et al, 2002) was found to reduce nitric oxide production (IC_{50} = 1.1 μM; Lucas et al, 2003). (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-7-Formidocycloamphilect-11(20)-ene (**185**) was from the sponge *C. hooperi* (Wright and Lang-Unnasch, 2009). (3*S**,4*R**,7*S**,8*S**,11*S**,13*S**)-8-Isocyanocycloamphilect-1(12)-ene (**186**) was isolated from the sponge *Halichondria* sp. and exhibited antibacterial activity against *S. aureus* and *B. subtilis* at 5 μg/mL in standard disk assay (Molinski et al, 1987).

(1*S*,3*S*,4*R*,7*S*,8*S*,11*S*,12*S*,13*S*,15*R*,20*R*)-7-Formamido-20-isocyanoisocycloamphilectane (**187**) was isolated from the sponges *Adocidae* sp. (Sharma et al, 1992), *Cymbastela hooperi* (Wright and Lang-Unnasch, 2009). (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-8-Isothiocyanatocycloamphilect-10-ene (**188**) and (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-8-isocyanatocycloamphilect-10-ene (**189**) were isolated from the sponge *Stylissa* sp. (Mitome et al, 2004). (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-8-Formamidocycloamphilect-10-ene (**190**) and (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-8,9-cyclicformamidocycloamphilect-10-ene (**191**) were isolated from the sponge *Axinella* sp. (Ciasullo et al, 2002), were reported to reduce nitric oxide production (IC_{50} = 0.2 and 0.6 μM, respectively; Lucas et al, 2003).



180 **181** **182** **183**



184 **185** **186** **187**

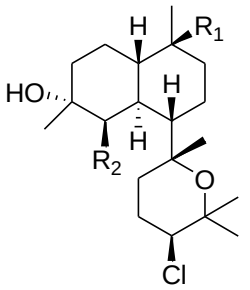
188 **189** **190** **191**

The kalihinane diterpenes are other exclusively marine-derived terpenes. The core structure of the kalihinanes comprises a decalin ring connected to either a tetrahydropyran or tetrahydrofuran ring. Most of kalihinane diterpenes are functionalized with up to three to five different functional groups, included among of which are the isonitrile-related functional groups, halide, and hydroxyl groups.

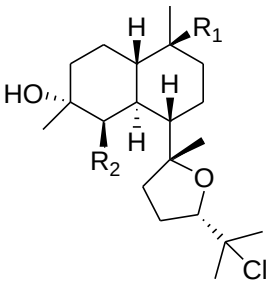
The prototype of kalihinanes, kalihinols A (**192**) was first isolated from the sponges *Acanthella cavernosa* (Omar et al, 1988; Okino et al, 1995; Okino et al, 1996b; Xu et al, 2012), *Acanthella* sp. (Miyaoka et al, 1998; White and Wood, 2001; Yan et al, 2006), *Ac. klethra* (Fusetani et al, 1990), *Phakellia pulcherrima* (Wolf and Schmitz, 1998), and the nudibranch *Phyllidiella pustulosa* (Manzo et al, 2004). The compound showed antifouling against barnacle *Balanus amphitrite* larvae (IC_{50} = 0.087 μg/mL; Okino et al, 1995), and antiplasmodial activity against *Plasmodium falciparum* FCR-3 (ATCC30932) (IC_{50} = 1.2 nM; Hirota et al, 1998). Kalihinol A analogs include 10β-formamidokalihinol A (**193**) and 10β-formamido-5β-isothiocyanatokalihinol A (**194**) [from the sponge *Ac. cavernosa;* Hirota et al, 1996; Xu et al, 2012), 10β-formamido-5β-isocyanatokalihinol A (**195**) [from the sponge *Ac. cavernosa* (Hirota et al,

1996)], have been isolated. All compounds showed antifouling activity against barnacle *Balanus amphitrite* larvae at 5 µg/mL (Hirota et al, 1996).

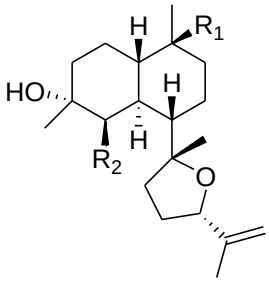
Other analogs of kalihinols include kalihinol B (**196**) [from the sponges *P. pulcherrima* (Wolf and Schmitz, 1998), and *Acanthella* sp. (White and Wood, 2001)], isokalihinol B (**197**) [from the sponges *Acanthella cavernosa* (Trimurtulu and Faulkner, 1994), *Ac. klethra* (Fusetani et al, 1990), kalihinol C (**198**) [from the sponges *Acanthella* sp. (White and Wood, 2001), and *Phakellia pulcherrima* (Wolf and Schmitz, 1998)], 10-isothiocyanatokalihinol C (**199**) [from the sponge *P. pulcherrima* (Wolf and Schmitz, 1998)], kalihinol D (**200**) [from the sponge *Acanthella* sp. (White and Wood, 2001)], kalihinol E (**201**) [from the sponges *Acanthella* sp. (White and Wood, 2001), *Ac. cavernosa* (Hirota et al, 1996; Xu et al, 2012), and the nudibranch *Phyllidiella pustulosa* (Manzo et al, 2004), 10β-formamidokalihinol E (**202**) [from the sponges *Acanthella* sp. (White and Wood, 2001), *Ac. cavernosa* (Hirota et al, 1996; Xu et al, 2012). Compound **197** showed cytotoxicity against P388 cancer cells (IC_{50} = 0.8 µg/mL; Fusetani et al, 1990), and compounds **200** and **201** showed antifouling activity against barnacle *Balanus amphitrite* larvae (IC_{50} = 5 µg/mL; Hirota et al, 1996).



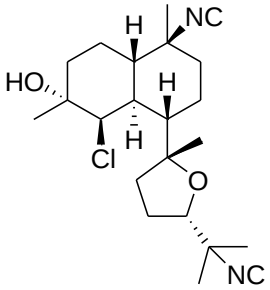
192 R_1=NC, R_2= NC
193 R_1= NHCHO, R_2= NC
194 R_1= NHCHO, R_2= NCS
195 R_1= NHCHO, R_2= NCO



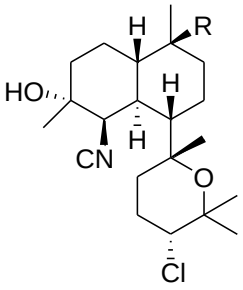
196 R_1= NC, R_2= NC
197 R_1= NC, R_2= OH



198 R_1= NC, R_2= NC
199 R_1= NCS, R_2= NC



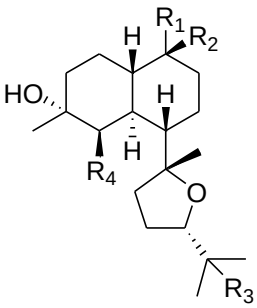
200



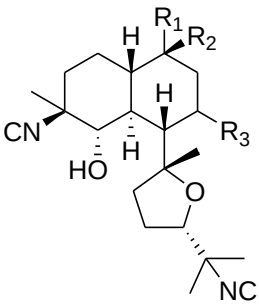
201 R= NC
202 R= NHCHO

The extended variation of the kalihinols both with tetrahydrofuran and with tetrahydropyran rings have been isolated, kalihinol F (**203**) [from the sponges *Acanthella cavernosa* (Omar et al, 1988; Bugni et al, 2004), *Acanthella* sp. (White and Wood, 2001), 10-formamidokalihinol F (**204**), and 15-formamidokalihinol F (**205**) [from the sponge *Ac. cavernosa* (Bugni et al, 2004)], isokalihinol F (**206**) from sponge *Ac. cavernosa* (Omar et al, 1988; Trimurtulu and Faulkner, 1994; Clark et al, 2000)], 8-hydroxyisokalihinol F (**207**) [from the sponge *Ac. cavernosa* (Clark et al, 2000)], and 10-*epi*-isokalihinol F (**208**) [from the sponge *Ac. cavernosa* (Trimurtulu and Faulkner, 1994)], kalihinols K (**209**) and L (**210**) [from the sponge *Phakellia pulcherrima* (Wolf and Schmitz, 1998)].

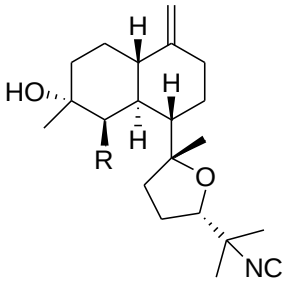
Kalihinols M (**211**) [from the sponge *Ac. cavernosa* (Xu et al, 2012)], kalihinol G (**212**) [from the sponges *Ac. cavernosa* (Bugni et al, 2004), *Acanthella* sp. (Hirota et al, 1998; White and Wood, 2001)], 10-isothiocyanatokalihinol G (**213**) [from the sponges *Phakellia pulcherrima* (Wolf and Schmitz, 1998), and *Ac. cavernosa* (Xu et al, 2012)], 5,10-bisisothiocyanatokalihinol G (**214**) [from the sponge *Acanthella* sp. (Hirota et al, 1998)], kalihinal H (**215**) [from the sponge *Acanthella* sp. (Miyaoka et al, 1998)], 10-*epi*-kalihinol H (**216**) [from the sponges *Ac. cavernosa* (Trimurtulu and Faulkner, 1994) and *P. pulcherrima* (Wolf and Schmitz, 1998)].



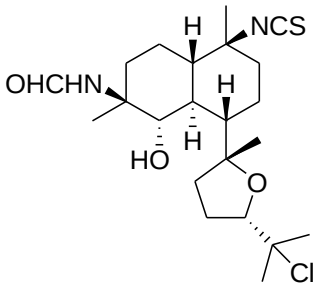
203 R_1= Me, R_2= NC, R_3= NC, R_4= NC
204 R_1= Me, R_2= NHCHO, R_3= NC, R_4= NC
205 R_1= Me, R_2= NC, R_3= NHCHO, R_4= NC
212 R_1= Me, R_2= NC, R_3= NCS, R_4= NC
213 R_1= NCS, R_2= Me, R_3= NCS, R_4= NC
214 R_1= Me, R_2=NCS, R_3= NCS, R_4= NCS
215 R_1= Me, R_2= NCS, R_3= NC, R_4= NC
216 R_1= NC, R_2= Me, R_3= NC, R_4= NC



206 R_1= Me, R_2= NC, R_3= H
207 R_1= Me, R_2= NC, R_3= OH
208 R_1= NC, R_2= Me, R_3= H



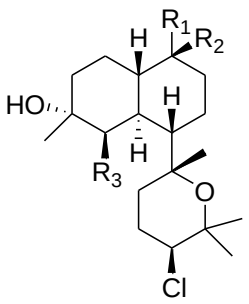
209 R= NC
210 R= NCS



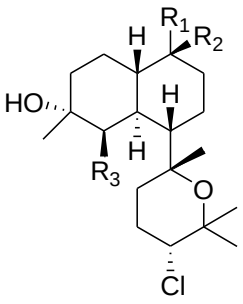
211

The list of kalihinols with tetrahydropyran extension include kalihinol I (**217**) [from the sponges *Acanthella* sp. (Miyaoka et al, 1998; White and Wood, 2001) and *Ac. cavernosa* (Xu et al, 2012)], 10-*epi*-kalihinol I (**218**) [from the sponges *Acanthella* sp. (Miyaoka et al, 1998) and *Ac. cavernosa* (Xu et al, 2012)], kalihinol J (**219**) [from the sponge *Ac. cavernosa* (Bugni et al, 2004)], and kalihinols N - T (**220** - **226**) [from the sponge *Ac. cavernosa* (Xu et al, 2012)], kalihinol X (**227**) [from the sponges *Ac. cavernosa* (Omar et al, 1988; Bugni et al, 2004), *Acanthella* sp. (White and Wood, 2001), and *Phakellia pulcherrima* (Wolf and Schmitz, 1998)], 10-*epi*-kalihinol X (**228**) [from the sponge *Ac. cavernosa* (Xu et al, 2012)], kalihinol Y (**229**) [from the sponges *Ac. cavernosa* (Omar et al, 1988; Bugni et al, 2004), *P.*

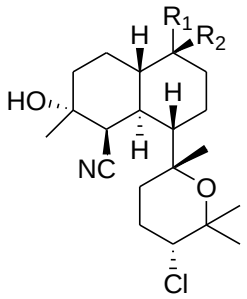
pulcherrima (Wolf and Schmitz, 1998)], Δ^9-kalihinol Y (**230**) [from the sponges *Acanthella* sp. (Hirota et al, 1998) and *Phakellia pulcherrima* (Wolf and Schmitz, 1998), kalihinol Z (**231**) [from the sponges *Ac. cavernosa* (Omar et al, 1988; Bugni et al, 2004), *Acanthella* sp. (White and Wood, 2001), and *P. pulcherrima* (Wolf and Schmitz, 1998).



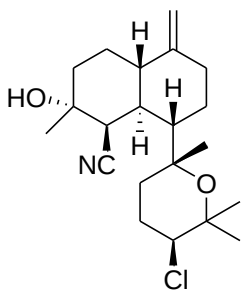
217 R_1= NCS, R_2= Me, R_3= NCS
218 R_1=Me, R_2= NCS, R_3= NCS
219 R_1= NCS, R_2= Me, R_3=NHCHO
222 R_1= Me, R_2= NC, R_3= NCS



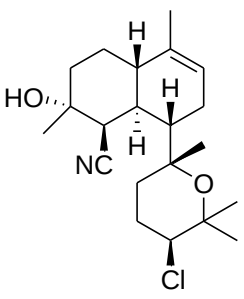
220 R_1= Me, R_2= NCS, R_3= OH
221 R_1= Me. R_2= NCS, R_3= NC
223 R_1= Me, R_2= NC, R_3= NCS
224 R_1= Me, R_2= NCS, R_3 NCS
225 R_1= Me, R_2= NCS, R_3= NHCHO
226 R_1= Me, R_2= NC, R_3= Cl



227 R_1= NCS, R_2= Me
228 R_1= Me, R_2= NCS
231 R_1= NC, R_2= Me



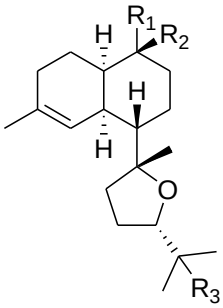
229



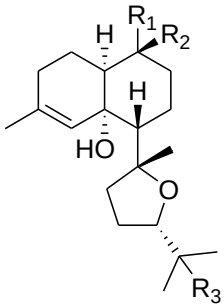
230

Among the kalihinols listed above, the active analogs include **203**, **209**, **212**, **203** and **209** showed antibacterial activity against *B. subtilis* PY79 (Bugni et al, 2004), and **212** showed antiplasmodial activity against *Plasmodium falciparum* FCR-3 (ATCC30932) (IC_{50} 2.6 μM; Hirota et al, 1998).

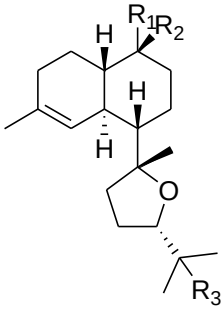
A series of kalihinanes with Δ^4 olefinic bond, hence the names kalihinene, were also reported. These include kalihinene (**232**) [from the sponges *Acanthella. klethra* (Fusetani et al, 1990), *Ac. cavernosa* (Rogríguez et al, 1994), and *Acanthella* sp. (Hirota et al, 1998), 10-formamidokalihinene (**233**) [from the sponge *Ac. cavernosa* (Rogríguez et al, 1994; Okino et al, 1995)], 15-formamidokalihinene (**234**) [from the sponge *Ac. cavernosa* (Rogríguez et al, 1994; Okino et al, 1996)], 10,15-diformamidokalihinene (**235**) [from the sponge *Ac. cavernosa* (Rogríguez et al, 1994)]. 6-Hydroxykalihinene (**236**) [from the sponges *Ac. cavernosa* (Rogríguez et al, 1994) and *Acanthella* sp. (Hirota et al, 1998), 6-hydroxy-10-formamido-kalihinene (**237**), 6-hydroxy-10-formamido-15-isothiocyanatokalihinene (**238**), and 6-hydroxy-15-formamidokalihinene (**239**) [from the sponge *Ac. cavernosa* (Rogríguez et al, 1994)]. 1,10-Di-*epi*-kalihinene (**240**), 1-*epi*-kalihinene (**241**), and 15-isothiocyanato-1-*epi*-kalihinene (**242**) [from the sponge *Ac. cavernosa* (Trimurtulu and Faulkner, 1994)], kalihinenes X (**243**), Y (**244**), and Z (**245**) [from the sponge *Ac. cavernosa* (Okino et al, 1995).



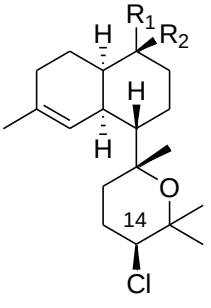
232 R_1= Me, R_2= NC, R_3= NC
233 R_1= Me, R_2= NHCHO, R_3= NC
234 R_1= Me, R_2= NC, R_3= NHCHO
235 R_1= Me, R_2= NHCHO, R_3= NHCHO



236 R_1= Me, R_2= NC, R_3= NC
237 R_1= Me, R_2= NHCHO, R_3= NC
238 R_1= Me, R_2= NHCHO, R_3= NCS
239 R_1= Me, R_2= NC, R_3= NHCHO



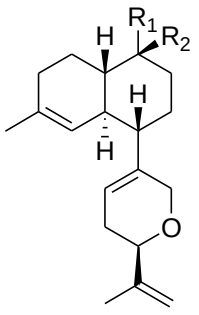
240 R_1= NC, R_2= Me, R_3= NC
241 R_1= Me, R_2= NC, R_3= NC
242 R_1= Me, R_2= NC, R_3= NCS



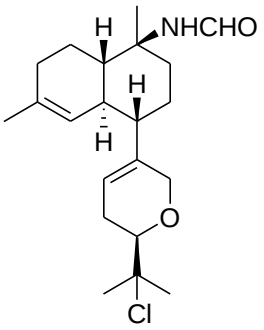
243 R_1= Me, R_2= NHCHO, 14βCl
244 R_1= Me, R_2= NHCHO, 14βCl
245 R_1= Me, R_2= NHCHO, 14αCl

Certain kalihinene series have also been examined for the biological activities. Compound **233** showed antibacterial activity against *B. subtilis* PY79 (Bugni et al, 2004), and showed antiplasmodial activity against *Plasmodium falciparum* FCR-3 (ATCC30932) (IC_{50} = 10 nM; Fusetani et al, 1990). Compounds **234**, **243**, **244**, and **245** showed antifouling activity against barnacle *Balanus amphitrite* larvae (IC_{50} = 0.14, 0.49, 0.45, and 1.1 µg/mL, respectively; Okino et al, 1995; Okino et al, 1996). Compound **236** also showed antiplasmodial activity against *P. falciparum* FCR-3 (ATCC30932) (IC_{50} = 80 nM; Fusetani et al, 1990).

Alternative oxygenation pattern over the tetrahydropyran ring yields the kalihipyran series. Three analogs, including kalihipyran (**246**), kalihipyrans A (**247**) and B (**248**), were isolated from the sponge *Acanthella cavernosa* (Trimurtulu and Faulkner, 1994; Okino et al, 1995; Bugni et al, 2004). Compounds **248** and **249** showed antifouling activity against barnacle *B. amphitrite* larvae (IC_{50}= 1.30 and 0.85 µg/mL, respectively; Okino et al, 1996b).



246 R_1= Me, R_2= NC
247 R_1= Me, R_2= NHCHO



248

1.5 8,15-Diisocyanoamphilect-11(20)-ene (DIA) and antiplasmodial activity

8,15-Diisocyanoamphilect-11(20)-ene (DIA; **150**) is an isonitrile diterpene, first isolated from the marine sponge *Hymeniacidon amphilecta* (Wratten and Faulkner, 1978). The compound was isolated from the specimen of *Stylissa* cf. *massa* sponge that was investigated in

this project and the strong antiplasmodial activity against *Plasmodium falciparum* K1 (IC_{50} = 0.09 μM) was published in the stated reported (Wattanapiromsakul et al, 2009).

Having two isonitrile functionalities substituted on C-8 and C-15, the isonitrile groups are hypothesized to play an important role in the antiplasmodial activity. Compared with the other diterpenes isolated from the specimen of *S. massa* investigated in the previous report (Wattanapiromsakul et al, 2009) and in this current one, other related functional groups, i.e., isothiocyanate and isocyanate groups may also exert their importance to a certain extent, however not as influential as the two isonitrile groups of DIA (see section 3.3). The effect of isonitrile group on the antiplasmodial activity may involve the formation of the complex with heme, hence preventing the biocrystallization of hemozoin, and allowing toxic free heme to be available in the cytosol of the parasites (see section 1.6). The complex formations between heme and small isonitrile molecules have been documented (Patel and Kassner, 1989; Vadon-Le Goff et al, 2001).

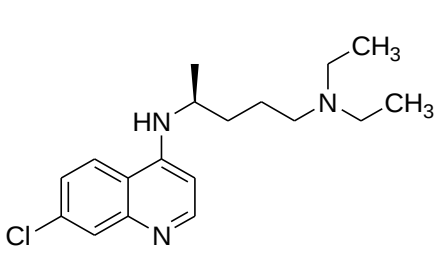
1.6 Heme as a target for antimalarial agents

Malaria is the infective diseases caused by *Plasmodium* parasites, including *P. falciparum*, *P. vivax*, *P. malariae*, *P.ovale*, and *P.knowlesi*. In 2013, an approximate of 3.4 billion peoples were at risk with malaria globally. As of 2012, 207 million cases and 627,000 deaths due to malaria were reported by WHO (WHO, 2013), with 80% of cases and 90% of deaths in Africa region, and 77% of deaths were children under five years old.

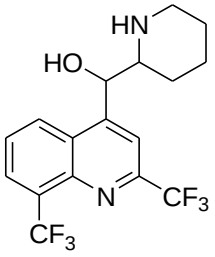
Among the pathogenic *Plasmodium*, specifically important in health care system were *P. falciparum* and *P*. vivax. *P. falciparum*, distributing mainly in Africa, Eastern Mediterranean, and South-East Asia, is the major cause of chloroquine- and multi-drugs resistant malaria. On the other hand, although less fatal, *P. vivax* is the main cause of relapsed malaria, due to its hibernating hypnozoite state in liver.

Several antimalarial drugs including sulfonamides and tetracyclines have been proved to have antiplasmodial effect, and some have been widely used. However, to date, only

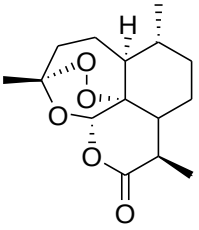
two classes of medicines, quinolines and artemisinins, are recommended by WHO as the effective antimalarial drugs. In fact, WHO specifically recommends the ACTs (artemisinin-based combination therapies) as an effective approach for the treatment of malaria. In general, this includes a loading dose of artesunate (4 mg/kg/day for 3 days), followed by a combination of artesunate and regionally effective quinolines for a specific period. For example, recommended for Thailand is a loading dose of artesunate (4 mg/kg) once a day for three days and 25 mg/kg of mefloquine either spliting over two days as 15 mg/kg and 10 mg/kg or over three days as 8.3 mg/kg/day once a day (WHO, 2010; WHO, 2013).



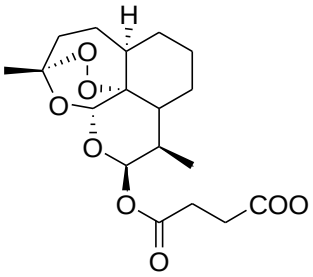
chloroquine



mefloquine



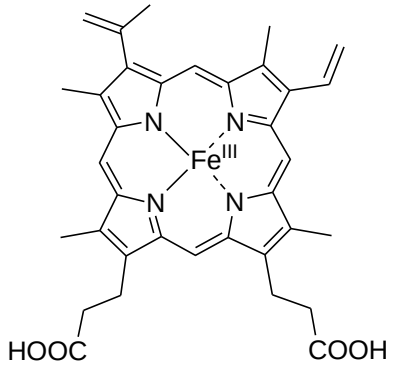
artemisinin



artesunate

Mechanistically, both artemisinins and quinolones have been proposed to target heme and its Fe(II)/Fe(III) status. *Plasmodium* parasites feed on hemoglobin in erythrocytes, digesting the globin protein as their carbon and nitrogen sources, and leaving heme as waste. However, having Fe(III) in the molecule, heme actually is a strong oxidizing agent and is highly toxic to all living cells. Whereas mammal cells cope with toxic heme through oxygenase family

enzymes, *Plasmodium* lack of such mechanism (Dorn et al, 1998). Heme detoxification processes in the *Plasmodium* parasites involve the conversion of heme to non-toxic, precipitating polymers called hemozoin. The polymerization of heme takes place in the parasitic acidic food vacuoles by several means, including biocrystalization and precipitation mediated by histidine-rich proteins and lipids. The remaining heme that may present in cytosol can be detoxified by complexation with glutathione or other detoxifying proteins before being discarded through peroxidation reaction (Kumar et al, 2007).



heme

Having an oxidative endoperoxide moiety, artemisinins react with Fe(II) in heme through single-electron oxidative cleavage, resulting in artemisinin–oxy radicals. The following 1,5-hydrogen radical shift (**a**) or oxidative cleavage (**b**) yield the toxic nucleophilic carbon radicals (Figure 2) (Chaturvedi et al, 2010).

On the other hand, quinoline antimalarial drugs react with heme through π-π stacking mechanism. Particularly susceptible for such complexation is the μ-oxo dimer of heme, which generally is an intermediate of hemozoin (Figure 3). Formation of quinoline-dimer adduct shunts the regular route of heme precipitation, yields the toxic free heme, and causes oxidative stress fatal to parasitic cells (Kuter et al, 2014).

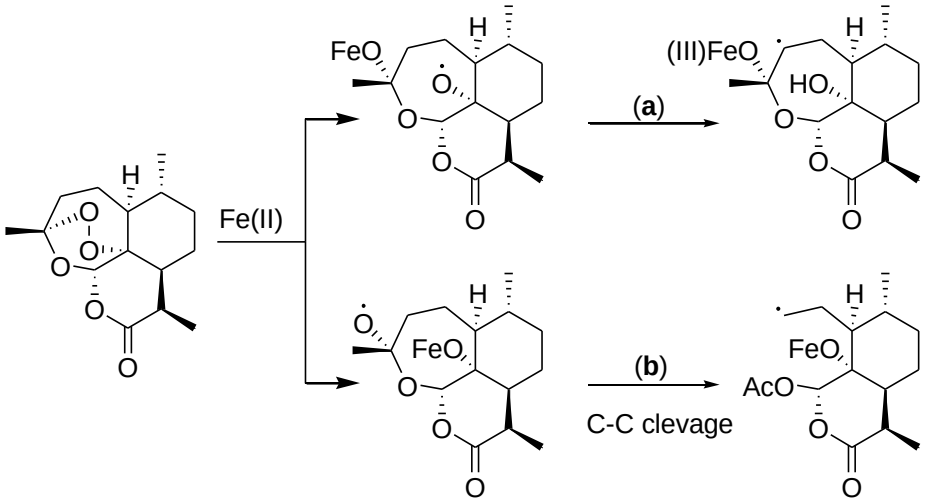


Figure 2. Carbon radical of artemisinin generated by Fe (II) in heme

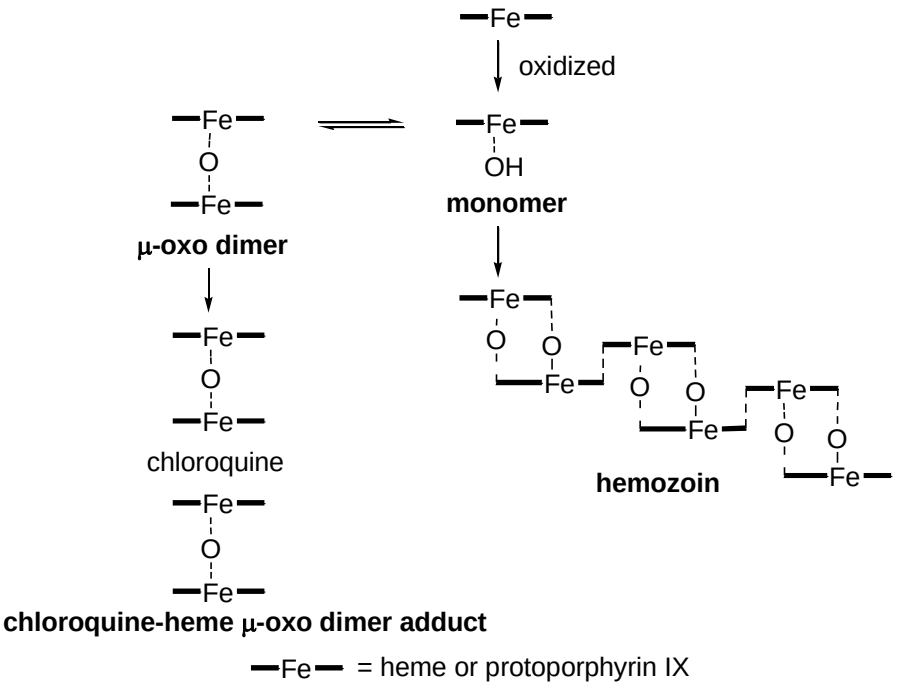


Figure 3. Complex formation of chloroquine-heme μ-oxo dimer adduct

1.7 Objectives

The dissertation "Bioactive constituents from the sponge *Stylissa* cf. *massa* and the soft coral *Eleutherobia* sp." presenting here combines two separated research projects on two different marine invertebrates, the sponge *S. massa* and the soft coral *Eleutherobia* sp. Both animal materials were previously screened for their biological activities. The sponge *S. massa* showed antimalarial activity against *Plasmodium falciparum* K1. The soft coral exhibited cytotoxicity toward KB cell line (human oral epidermoid carcinoma).

In addition, in line with the antiplasmodial isonitrile and related diterpenes isolated from *S. massa* sponge as mentioned earlier and also from the isonitrile diterpenes isolated in a previous work on *S. massa* (Wattanapiromsakul et al, 2009), the mechanistic aspect of the isonitrile diterpenes is therefore of interest. The complex formation between heme and DIA, representing here as the most active isonitrile diterpene in this family, is probed as a potential mechanism of the antiplasmodial activity.

The objectives of this study are

(i) To isolate the chemical constituents from the sponge *Stylissa* cf. *massa* and the soft coral *Eleutherobia* sp.

(ii) To elucidate the chemical structures of the isolated compounds from both the sponge *Stylissa* cf. *massa* and the soft coral *Eleutherobia* sp.

(iii) To determine the biological activities of the isolated compounds as stated in (ii).

(iiii) To study the characteristic features of complex formation of 8,15-diiso-cyanoamphilect-11(20)-ene (DIA), with heme and hemoglobin.

CHAPTER 2

EXPERIMENTAL

2.1 General

Unless stated otherwise, all of the chemicals were used as purchased without further purification. Chromatographic solvents were commercial graded and were re-distilled prior to use. HPLC solvents were HPLC graded, and were filtered through a 0.45 μm membrane filter then degassed by sonication prior to use. TLC was performed using silica gel F 60 (0.02 mm thickness) on aluminum support (Merck®). Detection was done under 254 nm, and with either iodine vapor or anisaldehyde/H_2SO_4 spraying reagent. Flash and vacuum chromatographies were performed on silica gel (mesh size 0.04-0.06 mm; Salicycle®). Size-exclusion chromatography was performed on Sephadex LH-20 (GE Healthcare®), saturated for an overnight with eluting solvents as stated.

HPLC was performed on a Waters® 1525 binary delivery system, equipped with a Waters® 2998 photodiode array detector and a Rheodyne® 7125i injector port. IR spectra were recorded on a Jasco® IR-810 infrared spectrophotometer or on a Perkin-Elmer® Spectrum One FT-IR spectrophotometer. Optical rotations were determined on a Perkin-Elmer® 341 polarimetor. UV-visible spectra were recorded either on a Genesys™6 UV-Visible spectrophotometer or on a UV SPECORD 205 spectrophotometer. Emission spectra were operated on a Cary Eclipse Varian® fluorescence Spectrophotometer. CD spectra were performed on a Jasco® PCT-423S J-810 spectropolarimeter. Mass spectra were reported either from a TOF Micromass® LCT mass spectrometer on an HR-TOF maXis® mass spectrometer.

NMR spectra were recorded either on an FT-NMR Varian® Unity Inova 500 spectrometer (500 MHz for ^{1}H, 125 MHz for ^{13}C), or on an FT-NMR DRX-400 Bruker® AVANCE spectrometer (400 MHz for ^{1}H). Operating NMR solvents were $CDCl_3$, benzene-d_6, DMSO-d_6, and D_2O as stated accordingly (Cambridge Isotope® and Euriso-Top®). NMR signals

were reported in chemical shifts, referencing solvent signal (7.24 ppm for residual $CHCl_3$ and 77.0 ppm for $CDCl_3$, 7.15 ppm for residual C_6D_5H and 128.0 ppm of C_6D_6, and 2.50 ppm for residual C_2D_5HSO and 39.5 ppm for C_2D_6SO).

2.2 Animal materials

2.2.1 The sponge *Stylissa* cf. *massa*

The sponge *Stylissa* cf. *massa*, (family Dictyonellidae, order Halichondrida; Carter, 1887) was collected by SCUBA from the vicinity of Koh-Tao, Surat Thani, Thailand (10° 7.569′ N, 99° 8.665′ E) at the depth of 15 - 20 m, in April, 2002. The taxonomic identification was kindly supported by Dr. Sumaitt Putchakarn, Marine Science Institute, Burapha University, Chonburi, Thailand. The specimen was stored in an ice chest upon surfacing and at -20°C once arrived at the lab. The voucher specimens are deposited at Marine Science Institute, Burapha University, Chonburi, Thailand (BIMS-I2001), and at Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand (AP02-006-02). The sponge is dark red to reddish brown with light khaki to yellow at the inner part. The texture is porous and soft but not fragile. The specimen turned white when soaking in the preserving solution (70% ethanol).

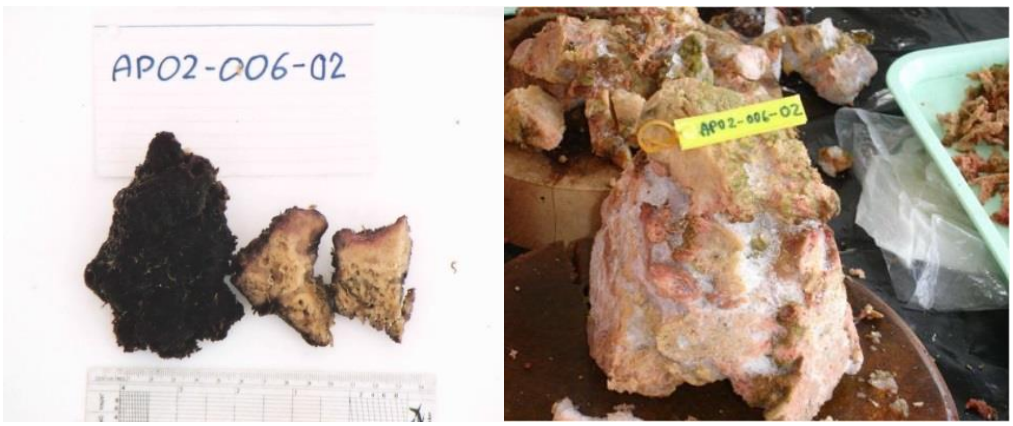


Figure 4. The sponge *Stylissa* cf. *massa*

2.2.2 The soft coral *Eleutherobia* sp.

The soft coral *Eleutherobia* sp. (family Alcyoniidae, order Alcyonacea) was collected by SCUBA from Koh-Ha Islets, Krabi, Thailand (7° 40.6′ N, 98° 37.7′ E) in May, 2011, at the depth of 25 - 30 m. The specimen was kept in an ice chest upon surfacing and at -20°C once arrived at the lab. The taxonomic identification was kindly performed by Dr. Thanongsak Chanmethakul, Department of Biology, Faculty of Science and Technology, Phuket Rajabhat University, Phuket, Thailand. The voucher specimen (AP11-008-03) is lodged at Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Songkhla, Thailand. The soft coral has a cylindrical shape (10 - 20 mm long and 0.3 - 0.5 mm wide), with yellow to orange color underwater which turns dark orange upon surfacing. Retractable white polyps were observed. The texture is slippery, leathery, and tough.

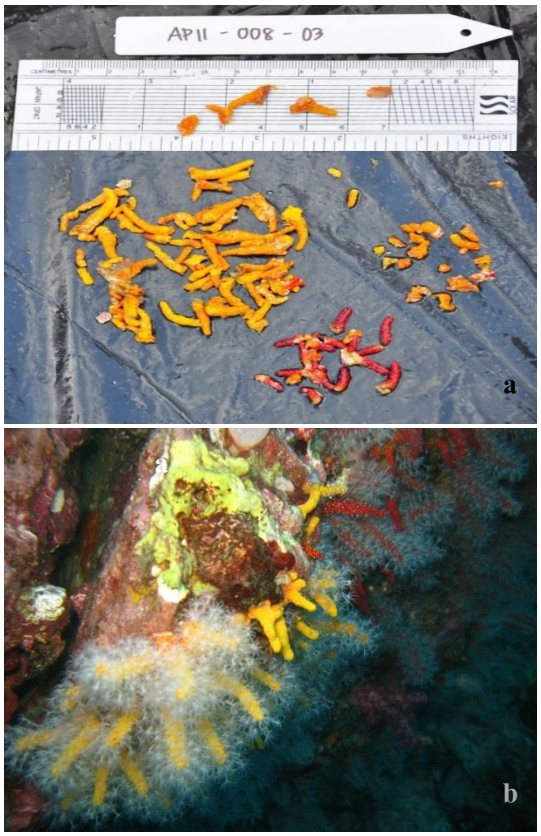


Figure 5. The soft coral *Eleutherobia* sp. (a) surface, (b) under water

2.3 The isolation and purification

2.3.1 Chemical investigation of the sponge *Stylissa* cf. *massa*

The freeze-dried sponge (297.0 g) was consecutively extracted with hexane (6 L × 5), dichloromethane (6 L × 5) and methanol (6 L × 5), to yield the extracts from the according solvents weighed 7.0 g, 6.0 g, and 63.0 g, respectively. The hexane extract, which showed a potent antiplasmodial activity (IC_{50} = 0.05 μg/mL), was isolated using flash chromatography (SiO_2; hexane to CH_2Cl_2 to CH_2Cl_2/MeOH 1:1). Two major fractions were pooled and investigated. Amphilectane-type diterpenes, 8,15-diisocyanoamphilect-11(20)-ene (DIA), 7-isocyanoamphilecta-11(20),15-diene, 8-isocyanoamphilecta-11(20),15-diene, and 8-isocyanoamphilecta-11(20),14-diene, were isolated from the more polar fraction, and had been reported previously (Wattanapiromsakul et al, 2009).

The other fraction was purified using Sephadex LH-20 (hexane/EtOAc 1:1), SiO_2 (hexane/EtOAc 1:1), and SiO_2 (hexane/EtOAc/THF 8:2:1) columns, leading to two sub-fractions. The more polar fraction was isolated with RP-C18 HPLC (VertiSep™, 10 μm, 10 × 250 mm; MeCN/H_2O 19:1, 3.0 mL/min) to yield compounds **A** (1.5 mg; 0.21%), **B** (4.7 mg; 0.66%), **C** (4.8 mg; 0.67%), and **D** (3.8 mg; 0.53%) at t_R 13.9, 15.4, 10.8, and 16.6 min, respectively.

8-Isocyanato-15-formamidoamphilect-11(20)-ene (A): viscous oil; $[\alpha]_D$ -49 (*c* 0.075, CH_2Cl_2); UV (CH_2Cl_2) λ_{max} (log ε) 224 (2.07) nm; IR (thin film) ν_{max} 3290, 2900, 2250, 1670 cm^{-1}; ^1H and ^{13}C NMR see Table 2; ESIMS *m/z* (% relative intensity) 381 ($[M+Na]^+$, 40), 359 ($[M+H]^+$, 100), 316 ($[M-NCO]^+$, 18); HRESIMS *m/z* 359.2713 calcd. for $C_{22}H_{35}N_2O_2$ 359.2698.

8-Isothiocyanato-15-formamidoamphilect-11(20)-ene (B): viscous oil; $[\alpha]_D$ -17 (*c* 0.27, CH_2Cl_2); UV (CH_2Cl_2) λ_{max} (log ε) 224 (2.30) nm; IR (thin film) ν_{max} 3280, 2900, 2075, 1660 cm^{-1}; ^1H and ^{13}C NMR see Table 3; ESIMS *m/z* (% relative intensity) 397 ($[M+Na]^+$, 12), 375 ($[M+H]^+$, 100); HRESIMS *m/z* 375.2474 calcd. for $C_{22}H_{35}N_2OS$ 375.2470.

8-Isocyano-15-formamidoamphilect-11(20)-ene (C): viscous oil; $[\alpha]_D$ -22 (*c* 0.19, CH_2Cl_2); UV (CH_2Cl_2) λ_{max} (log ε) 224 (2.36) nm; IR (thin film) ν_{max} 3250, 2960, 2130, 1670 cm^{-1}; ^1H and ^{13}C NMR see Table 4; ESIMS *m/z* (% relative intensity) 365 ($[M+Na]^+$, 75), 343 ($[M+H]^+$, 10), 316 $[M-NC]^+$, 100).

7-Formamidoamphilecta-11(20),15-diene (D): viscous oil; $[\alpha]_D$ +13 (*c* 0.12, CH_2Cl_2); UV (CH_2Cl_2) λ_{max} (log ε) 224 (2.51) nm; IR (thin film) ν_{max} 3275, 2950, 1640 cm^{-1}; ^1H and ^{13}C NMR see Table 5; ESIMS *m/z* (% relative intensity) 316 ($[M+H]^+$, 30), 271 ($[M-NHCHO]^+$, 100).

2.3.2 Chemical investigation of the soft coral *Eleutherobia* sp.

The soft coral *Eleutherobia* sp. (276.0 g wet weight) were chopped and macerated in EtOAc/MeOH 1:1 (500 mL × 10) to yield a crude extract (3.2 g). The further fractionation with solvents in gradually increasing polarity yield the extracts from hexane (1.4 g), CCl_4 (572 mg), $CHCl_3$ (475 mg), *n*-BuOH (270 mg), and H_2O (282 mg).

The chloroform extract, which showed the cytotoxicity against KB cancer cell line (66% inhibition), was selected for the further purification. The extract was subjected to Sephadex LH-20 (MeOH) column to yield three pooled fractions. The first fraction (260 mg) was further isolated with SiO_2 HPLC (VertiSep™; 5 μm, 4.6 × 250 mm; hexane/*i*-PrOH 87:13, 3.0 mL/min), then RP-C18 HPLC (VertiSep™; 5 μm, 4.6 × 150 mm; MeCN/H_2O 83:17, 1.2 mL/min), to yield compound **E** (1.0 mg, t_R 12.0 min). The second fraction (19.3 mg) was purified with RP-C8 HPLC (Ascentis™; 10 μm, 10 × 250 mm; MeCN/H_2O 19:1, 5.0 mL/min), and compounds **F** (2.7 mg, t_R 5.7 min) and **G** (9.3 mg, t_R 16.5 min) were obtained. Additional amount of **F** (2.4 mg, t_R 12.5 min) was also obtained from the last fraction by means of SiO_2 HPLC (VertiSep™; 5 μm, 4.6 × 250 mm; hexane/*i*-PrOH 4:1, 1.0 mL/min).

Xeneloide A (E): viscous oil; $[\alpha]_D$ -1.14 (*c* 0.10; $CHCl_3$); UV ($CHCl_3$) λ_{max} (logε) 268 (2.76) nm; IR (thin film) ν_{max} 3400, 2900, 1720 cm^{-1}; ^1H and ^{13}C NMR see Table 6; ESIMS *m/z* (% relative intensity) 355 ($[M+Na]^+$, 100), 304 (90).

Thymine (F): white solid; UV (MeOH) λ_{max} (logε) 209 (2.90), 263 (2.85) nm; ^1H and ^{13}C NMR see Table 7. HRESIMS *m/z* (% relative intensity) 527.1639 ($[4M+Na]^+$, 100), 149.0331 ($[M+Na]^+$, 56) calcd. for $C_5H_6N_2O_2Na$ 149.0327.

2*H*,5*H*,7*H*,9*H*-9-Hydroxy-imidazole[1,5-*a*]pyridine-1,3-dione (G): white solid; UV (MeOH) λ_{max} (logε) 216 (2.50) nm; IR (thin film) ν_{max} 3440, 2900, 1771, 1690 cm^{-1}; ^1H and ^{13}C NMR see Table 8; HRESIMS *m/z* (% relative intensity) 336.1296 ($[2M+Na]^+$, 100), 193.0596 ($[M+Na]^+$, 57) calcd. for $C_7H_{10}N_2O_3Na$ 193.0589.

2.4 The biological activity determination

2.4.1 Antiplasmodial activity

The antiplasmodial activity determination was serviced by BIOTEC Center Research Unit, BIOTEC Thailand, using a microculture radioisotope technique against *Plasmodium falciparum* K1 (Wangchuk et al, 2010; Panseeta et al, 2011). The parasites were cultured with human erythrocyte in RPMI 1640 medium, containing 25 mM *N*-(2-hydroxyethyl) piperazine-*N*-ethylethane sulfonic acid (HEPES), and supplemented with 0.2% $NaHCO_3$, 40 µg/mL gentamicin, and 10% human serum (Trager and Jensen, 1976; Wangchuk et al, 2002). The tested compounds were dissolved in DMSO and diluted with culture medium to the required final concentrations. To a 96-well plate was added 25 µL of tested samples, 200 µL of 1.5% parasitized erythrocyte cell suspension (1 - 2% parasitemia; final DMSO < 0.1%). This was incubated at 37°C (3% CO_2, 20% O_2) for 24 hours. [^{3}H]Hypoxanthine (25 µL, 0.25 µCi) was added, and the plate was incubated for additional 18 - 24 hours. At the end of the incubation time, the parasitic DNA was collected though glass-filter membrane, and allowed to air-dry.

Scintillation fluid (20 µL) was added. The radioactivity was measured by TopCount NXT microplate scintillation and luminescence counter. The activity in IC_{50} was determined from dose-response curve and referred to dihydroartemisinin and mefloquine (IC_{50} = 1.4 and 37.3 nM, respectively) (Desjadins et al, 1979; Wangchuk et al, 2002).

2.4.2 Antiproliferative activity

The antiproliferative activity determination was kindly supported by Assist. Prof. Dr. Supreeya Yuenyongsawad, Department of Pharmacognosy and Pharmaceutical Botany, Faculty of Pharmaceutical Scienes, Prince of Songkla University, Hat-Yai, Songkhla, Thailand, using sulforhodamine B colorimetric assay (Skehan et al, 1990). All the isolated compounds from both the sponge and the soft coral were evaluated for their cytotoxicity targeting MCF-7 (human breast adenocarcinoma), KB (human oral epidermoid carcinoma) and HeLa (human cervical carcinoma) cell lines, and referencing camptothecin as the reference standard.

The monolayered targeted cells in a 96-well microplate were incubated for 6 day at 37°C (5% CO_2 and 90% humidity) with five concentrations of each tested samples in EMEM culture medium (GIBCO®) containing 2 mM glutamine and 10% heat-inactivated fetal bovine serum, supplemented with 50 IU/mL penicillin G sodium, 50 µg/mL streptomycin sulphate and 0.125 µg/mL amphotericin B. The medium was refreshed at mid-way of this incubation. At the end of incubation period, cells were fixed with 100 µL of iced-cold 40% TCA for an hour. The cells were washed with tap water, and strained with 0.4% (w/v) sulforhodamine B in 1% acetic acid (Sigma-Aldrich®). After washing with 1% acetic acid, the microplate was air-dried (24 h). The dye was extracted with 100 µL of 10 mM Tris base (pH 10) (Sigma-Aldrich®). The optical density was measured at 492 nm on an PowerWave™ X Microplate Reader (BIO-TEK®). The IC_{50} was calculated based on dose-dependent curve.

2.5 Heme-8,15-diisocyanoamphilecta-11(20)-ene complex

2.5.1 Sample preparation

Hematin was used for the preparation of heme complex throughout this investigation. Upon complex formation, hematin transforms in situ to heme through losing its hydroxyl group (Egan et al, 2000).

Complex between 8,15-diisocyanoamphilect-11(20)-ene (DIA) and heme was prepared as followed. Stock solutions of hematin and DIA were separately prepared by dissolving an accurate amount of either hematin or DIA in DMSO. An appropriate amount of hematin solution was mixed with an acquired amount of DIA solution. The mixture was diluted quantitatively with 50%aq DMSO to a series of concentrations of DIA and heme as stated accordingly.

The complex formation between DIA and hemoglobin (Hb) was prepared in the same manner as that for DIA-heme complex. The solution of Hb was prepared as an aqueous solution. Bovine hemoglobin (90% identity to human hemoglobin; Yan et al, 2013) was used. The mixtures were quantitatively diluted with water to a series of acquired concentration of Hb (DMSO not exceed than 10%).

2.5.2 Spectroscopic measurement

Solutions of hematin (5 μM) and heme-DIA complex in a series of molar ratios (hematin/DIA 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, and 1:9), equivalent to 5 μM final concentration of heme in 50%aq DMSO, were subjected to the measurement of UV-visible spectra in an absorption range of 300 - 800 nm. In a similar manner, CD and emission spectra (excitation at 402 nm, emission range 460 - 780 nm) of heme and heme-DIA complex (100 μM and 15 μM of heme for CD and for emission spectra measurement, respectively) were measured. The molar ratio of heme-DIA complex for the measurement of the CD spectra was 1:20 of heme/DIA, and those for the emission spectra were 1:0.5, 1:1, 1:2, 1:4, and 1:20 of heme/DIA.

As for the NMR spectra, sample preparation was conducted in a similar manner to those stated above excepted that the deuterated solvents (DMSO-d_6 and D_2O) were used instead of the protonated ones. The measurement was performed at 400 MHz for ^1H, referred to the signal of HOD at 4.80 ppm.

Mass spectra of the heme-DIA complex were measured in an HR-ESI mode. The molar ratios of the heme-DIA complex were 1:0.5, 1:1, 1:2, 1:4, and 1:20 of heme/DIA.

The mixtures of hemoglobin (Hb)-DIA complex were also subjected to UV-visible absorption, CD, and emission spectral measurements and molar ratio for heme and DIA were 1:0.1, 1:0.5, 1:1, 1:2, 1:4, 1:10, and 1:20.

CHAPTER 3

RESULTS AND DISCUSSIONS

Two marine invertebrates, the sponge *Stylissa* cf. *massa* and the soft coral *Eleutherobia* sp., were chosen for the chemical investigation due to their biological activities. The extracts from *S. massa* showed antiplasmodial activity (IC_{50} = 0.05 μg/mL, against *Plasmodium falciparum* K1), and that from *Eleutherobia* sp. was active in cytotoxic assay against KB and HeLa cell lines (66% and 30% inhibition, respectively). The structure elucidations of all the isolated compounds, beginning with the newly reported followed by the known ones, are discussed here. In addition, in line with the previous study in which 8,15-diisocyanoamphilect-11(20)-ene (DIA) from *S. massa* was found strongly antiplasmodially active, the spectroscopic characterization of heme-DIA complex, potentially proposed as the mechanism of the antiplasmodial activity, is also discussed.

3.1 Isolation and structure determination of compounds from the sponge *Stylissa* cf. *massa*

3.1.1 Isolation and purification

The hexane extract of *S. massa* was subjected to a series of chromatographic separation, including Sephadex LH-20 (hexane/EtOAc 1:1), SiO_2 (hexane/EtOAc 1:1), SiO_2 (hexane/EtOAc/THF 8:2:1), and RP-C18 HPLC (MeCN/H_2O 9:1), and four compounds, **A**, **B**, **C**, and **D**, were obtained (0.21%, 0.66%, 0.67%, and 0.53%, respectively).

3.1.2 Structure determination

3.1.2.1 Compound A

Compound **A** (1.5 mg; 0.21% yield), was obtained as a yellow oil. The molecular formula of **A** was $C_{22}H_{34}N_2O_2$, as deduced from the $[M+H]^+$ ion peak at *m/z* 359, and was confirmed by HR-ESIMS at *m/z* 359.2713 (calcd. for $C_{22}H_{35}N_2O_2$ 359.2698). This leads to an

unsaturation degree of 7 attributed to three rings, one exomethylene, one isocyanate, and one formamide. An IR absorption band at ν 2250 cm^{-1} indicated the presence of an isocyanate functional group, and the bands at ν 3290 and 1670 cm^{-1} did the secondary amine and an amide carbonyl. The presence of the formamide moiety was indicated by the ^{13}C NMR spectrum (Figure 7, Table 2), which showed a formyl resonance at δ_C 159.6 (C-22), and also by the $[M\text{-}NCO\text{-}NH_2CHO]^+$ peak at *m/z* 271 in the mass spectrum. The isocyanate resonates at δ_C 123.0 (C-21), and showed an $[M\text{-}NCO]^+$ peak at *m/z* 316.

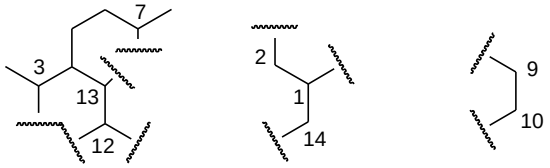
From the ^1H and ^{13}C NMR spectra (Figures 6 and 7, Table 2), two sets of resonances were observed in a 3:2 ratio. This was attributed to two rotameric conformers caused by slowly rotating formamide moiety. The major signal of formyl proton in ^1H NMR spectrum belonged to the *cis* conformer, which resonated more upfield than the minor signal and showed a small coupling constant (δ_H 7.68; d, *J*= 1.8 Hz). The minor signal was from the *trans* conformer, which showed downfield chemical shift and a large coupling constant (δ_H 8.17; d, *J*= 12.1 Hz). For brevity, the discussion here after is focusing on the major conformer. The chemical shifts of the minor one are bracketed in Table 2.

The ^{13}C NMR spectrum of **A** showed the resonances of 22 carbons of major signals, defined as four quaternary carbons, seven methines, seven methylenes, and four methyls in the DEPT experiments. In the ^1H NMR spectrum, the characteristic chemical shifts at δ_H 7.68 (d, *J*= 12.1 Hz, H-22), and 4.05 (br s, NH), which correlated to a formamide carbon at δ_C 159.6 (C-22), indicated the formamide moiety of compound **A**. The ^1H NMR signals at δ_H 4.81 (s, H-20a) and 4.68 (s, H-20b), which connected to an olefinic carbon at δ_C 106.0 (C-20) in the HMQC spectrum and showed a correlation to another olefinic carbon at δ_C 150.9 (C-11) in the HMBC spectrum, indicated the presence of an exomethylene in compound **A**. Four methyl groups were observed at δ_H 1.26 (s, H-16), 1.22 (s; H-17), 0.79 (d, *J*= 5.6 Hz; H-18), and 0.75 (d, *J*= 6.3 Hz; H-19). The remaining signals of methines and methylenes in the ^1H NMR spectrum clustered densely in the high-field region (δ_H 2.11 – 0.52). The structure determination of **A** therefore

relied heavily on the analysis of the COSY and HMBC correlations. Three spin systems, fragments **a** - **c**, can be clearly deduced from the COSY spectrum of **A**.

Fragment **a** is composed of four consecutive methines, starting from δ_H 1.68 (br d, *J*= 10.9 Hz; H-12), to 0.69 (br d, *J*= 13.9 Hz; H-13), 0.93 (overlap; H-4), and 0.79 (overlap; H-3). Placed onto C-4 is an ethylene bridge of δ_H 1.76 (overlap; H-5a), 0.52 (m; H-5b), 1.32 (m; H-6a), and 1.21 (overlap; H-6b), which connected to the H-7 methine at δ_H 0.90 (overlap; H-7). Two doublet methyls at δ_H 0.79 (d, *J*= 5.6 Hz; 3-CH_3) and 0.75 (d, *J*= 6.3 Hz; 7-CH_3) substitute on C-3 and C-7, respectively.

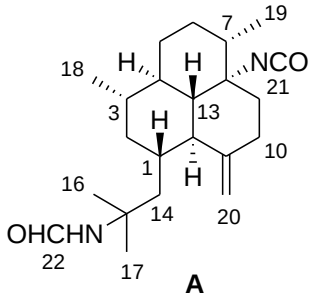
Fragment **b** is composed of the two methylenes at δ_H 1.80 (overlap, H-2a), 0.60 (m, H-2b), and 2.01 (br d, *J*= 13.6 Hz, H-14a), both of which are connected to a methine at δ_H 1.66 (overlap; H-1). Fragment **c** is an ethylene bridge resonating at δ_H 1.84 (overlap; H-9a), 0.94 (overlap; H-9b), 2.11 (ddd, *J*= 13.4, 13.1, 4.6 Hz; H-10a), and 1.98 (overlap; H-10b).



a **b** **c**

Connection of fragments **a**, **b**, and **c** was accomplished by means of the HMBC analysis. The correlations from δ_H 0.79 (H-3) to δ_C 41.2 (C-2), and from δ_H 1.66 (H-1), 2.01 (H-14a), and δ_H 1.20 (H-14b) to δ_C 47.0 (C-12) allowed the connection of fragments **a** and **b**, whereas those from δ_H 1.84 (H-9a), 0.94 (H-9b), and 0.75 (H-19) to δ_C 65.7 (C-8), and from δ_H 2.11 (H-10a), 1.98 (H-10b), and 1.68 (H-12) to δ_C 150.9 (C-11) did fragments **a** and **c**. The geminal methyl groups of δ_H 1.26 (H-16) and δ_H 1.22 (H-17) were placed on C-15 (δ_C 53.6) as indicated in the HMQC spectrum. C-15 further connected to δ_C 45.4 (C-14), δ_C 29.1 (C-16), and δ_C 27.7 (C-17). On the other hand, the terminal exomethylene was placed on C-11 (δ_C 150.9) according to the correlation from δ_H 4.81 (H-20a) and 4.68 (H-20b) to δ_C 150.9 (C-11) and 47.0

(C-12). An isocyanate group was placed on C-8 due to the characteristic chemical shift of the isocyanate-bearing carbon of C-8 (δ_C 65.7). In a similar manner, the formamide was placed on C-15 according to the chemical shift of C-15 at δ_C 53.6. The formyl proton (δ_H 7.68; H-22) also showed the correlation to C-15 in the HMBC spectrum. The structure of **A** was therefore proposed as a new amphilectane diterpene containing a formamide and isocyanate units, named 8-isocyanato-15-formamidoamphilect-11(20)-ene.



A

The relative configuration of **A** was determined using a series of nOe-ds experiments and also by the analysis of proton coupling constants. Despite clustering densely, the large coupling constants of the methines H-12 (δ_H 1.68, br d, *J*= 10.9 Hz) and H-13 (δ_H 0.69, br d, *J*= 13.9 Hz) can be measured. Such coupling constants indicated that both are axial, and presumably reside on the opposite planes. nOe Enhancements relevant to H-12, including those to δ_H 0.75 (H-19), 1.21 (H-6b), and 1.98 (H-10b), suggest that all resides on the same plane. On the other hand, the enhancements relevant to δ_H 1.32 (H-6a), which include 0.52 (H-5b), 1.80 (H-2a), and 0.79 (H-3), indicate that they are on the other side. Additional, enhancement between the protons at δ_H 1.76 (H-5a) and 0.79 (H-18) indicates that the two protons are on the same plane as that of H-6b. The relative configuration of the perhydrophenalene unit is therefore proposed to be all *trans* (Figure 8). The orientation of the isobutyl side chain was proposed to be on equatorial on the α plane as opposite to the axial proton of H-1 (δ_H 1.59, br dd, *J*= 10.9, 10.7 Hz). The relative configuration of all the seven asymmetric carbons are 1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**.

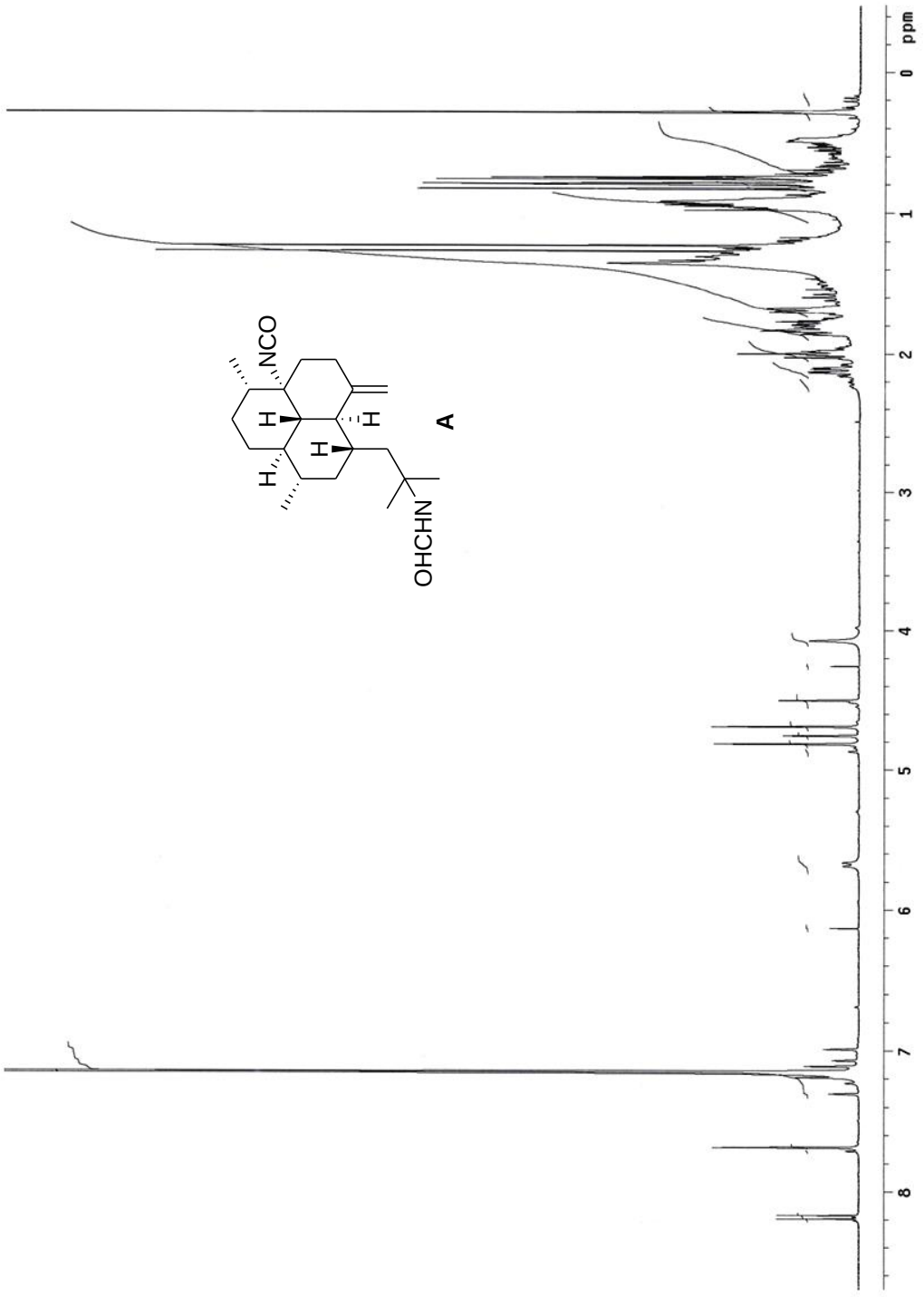


Figure 6. ^{1}H NMR spectrum of **A** (500 MHz, C_6D_6)

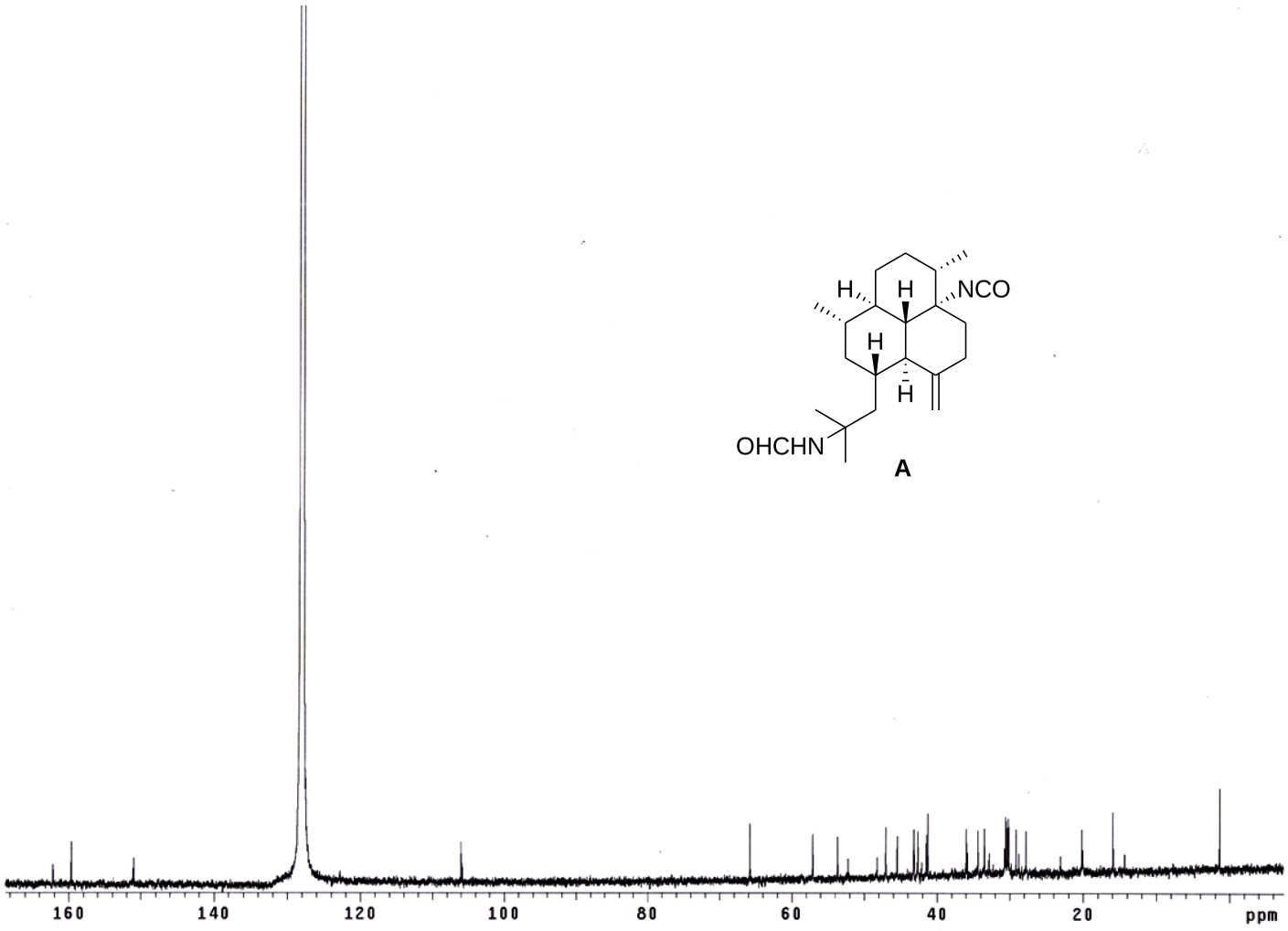


Figure 7. ^{13}C NMR spectrum of **A** (125 MHz, C_6D_6)

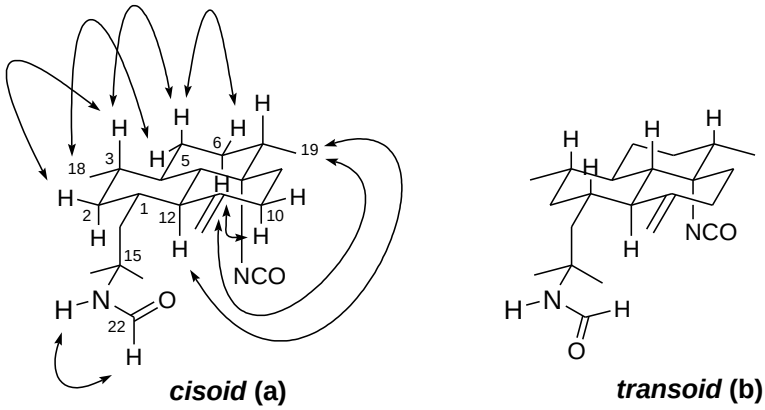


Figure 8. nOe Correlation of compound **A** in (a) cisoid and (b) transoid forms

The geometry of formamide moiety as *cisoid* for the major conformer and *transoid* for minor one was determined based on the coupling constant between the formyl and amide protons as stated earlier. This was also confirmed by the nOe enhancement between δ_H 7.68 (br d, *J*= 1.8 Hz; 22-NHC*H*O) and 4.05 (br s; 22-N*H*CHO) observed in the major conformer (Figure 8). Such enhancement was absent in the resonances assigned to the minor *transoid* conformer.

Table 2. NMR data of A (500 MHz for ^{1}H and 125 MHz for ^{13}C; C_6D_6)

position	^{13}C (mult)		^{1}H (*J* in Hz)	HMBC correlation (H→C)
1	33.4 (CH)	[32.8]	1.66, overlap	C-2, C-12
			[1.59, br dd, 10.9, 10.7]	
2a	41.2 (CH_2)		1.80, overlap	C-1
b			0.60, m	
3	35.9 (CH)	[35.8]	0.79, overlap	C-2
4	43.1 (CH)		0.93, overlap	C-13, C-18
5a	30.1 (CH_2)		1.76, overlap	
b			0.52, m	
6a	30.5 (CH_2)	[30.3]	1.32, m	C-5
b			1.21, overlap	

Table 2, (cont.)

position	^{13}C (mult)		^{1}H (*J* in Hz)	HMBC correlation (H→C)
7	42.6 (CH)	[42.5]	0.90, overlap	C-6
8	65.7 (C)			
9a	41.4 (CH_2)		1.84, overlap	C-20
b			0.94, overlap	
10a	34.3 (CH_2)		2.11, ddd (13.4, 13.1 4.6)	C-8, C-9, C-11, C-12
b			1.98, overlap	
11	150.9 (C)	[151.1]	-	
12	47.0 (CH)		1.68, br d (10.9)	C-11
13	57.0 (CH)		0.69, br d (13.9)	C-12, C-19
14a	45.4 (CH_2)	[48.2]	2.01, br d (13.6)	C-2, C-12, C-15, C-16,
b			1.20, overlap	C-17
15	53.6 (C)	[52.2]		
16	29.1 (CH_3)	[28.7]	1.26, s	C-14, C-15, C-17
17	27.7 (CH_3)	[30.7]	1.22, s	C-14, C-15, C-16
18	20.1 (CH_3)	[20.0]	0.79, d (5.6)	C-2, C-3, C-4
19	15.9 (CH_3)	[14.3]	0.75, d (6.3), [0.74, d, 6.3]	C-6, C-7, C-8
20a	106.0 (C)	[105.8]	4.81, s, [4.75, s]	C-10, C-11, C-12
b			4.68, s, [4.50, s]	
21-N*C*O	123.0 (C)			
22-NH*CH*O	159.6 (CH)	[162.1]	7.68 (br d, 1.8),	C-15
			[8.17, d, 12.1]	
22-N*H*CHO			4.05 (br s), [5.67, d, 12.1]	

Note; the chemical shifts of the minor conformer are presented in brackets.

3.1.2.2 Compound B

Compound **B** (4.7 mg; 0.66%) was obtained as a yellow viscous oil. The molecular formula of **B** was proposed to be $C_{22}H_{34}N_2OS$ as deduced from the pseudomelecular

peak of $[M+H]^+$ at *m/z* 375 in ESI-mass spectrum. This was confirmed by HR-ESI mass at *m/z* 375.2474 (calcd. for $C_{22}H_{35}N_2OS$ 375.2470). The unsaturation degree of 7 was deduced to be three rings, one exomethylene, one isothiocyanate, and one formamide. The IR spectrum showed the significant absorption bands at ν 3280 cm^{-1} and 1660 cm^{-1} similar to those of compound **A**, these suggesting the amide functionality. The band of isocyanate observed in compound **A** shifted to ν 2075 cm^{-1} and suggested the presence of an isothiocyanate group in compound **B**.

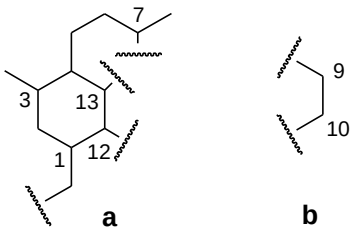
The ^1H and ^{13}C NMR spectra of compound **B** (Figures 9 and 10, Table 3) showed two sets of signals in a 3:2 ratio, which was attributed to the rotating formamide functionality similar to that of compound **A**. The formamide protons of the major conformer were observed at δ_H 7.69 (d, *J*= 1.7 Hz; 22-NHC*H*O) and 4.06 (br s; 22-N*H*CHO), and those of the minor one were at δ_H 8.16 (d, *J*= 12.1 Hz; 22-NHC*H*O) and 5.68 (d; *J*= 12.1 Hz; 22-N*H*CHO). The carbon counterparts resonated at δ_C 159.6 (C-22) for the major conformer, and at δ_C 162.1 (C-22) for the minor one.

In the same manner as that for **A**, the structure elucidation of **B** described here refers to the resonances of the major conformer. The chemical shifts of the minor conformer are bracketed in Table 3.

Twenty-two carbons signals were observed in the ^{13}C NMR spectrum and were identified to be seven methines, seven methylenes, four methyls, and four quaternary carbons, based on the DEPT experiments. Apart from the spin system of the formamide moiety describe above, two vinyl protons at δ_H 4.79 (4.72, s; H-20a) and 4.70 (4.49, s; H-20b) were observed in the ^1H NMR spectrum. These correlated to the carbons resonating at δ_C 106.5 (C-20) and 150.2 (C-11). The ^{13}C NMR spectrum also showed the characteristic signal of an isothiocyanate carbon resonating at δ_C 130.0.

Connecting the aliphatic methines and methylenes clustering in the high-field region was assisted through the COSY experiment, from which two unambiguous fragments can be identified. Fragment **a** is composed of a six-membered carbocyclic moiety comprising a spin

system of δ_H 1.65 (overlap; H-1) to δ_H 1.78 (overlap; H-2a), 0.62 (ddd, *J*= 14.6, 10.0, 3.4 Hz; H-2b), 0.76 (overlap; H-3), 0.94 (overlap; H-4), (1.69, br d, *J*= 10.6 Hz; H-12), and 0.70 (br dd, *J*= 10.6, 10.0 Hz; H-13). Extended from C-4 was a spin system of δ_H 1.75 (overlap; H-5a), 0.48 (m; H-5b), 1.31 (br dd, *J*= 13.4, 3.9 Hz; H-6a), 1.22 (overlap; H-6b), 0.92 (overlap; H-7), and 0.75 (d, *J*= 6.1 Hz; H-19). On the other ends, a methylene of δ_H 2.02 (dd, *J*= 14.6, 1.7 Hz; H-14a) and 1.02 (dd, *J*= 14.6, 10.0 Hz; H-14b) connected to C-1, and a methyl at δ_H 0.76 (d, *J*= 5.8 Hz; H-18) did to C-3. Another fragment, fragment **b**, is composed of a simple ethylene bridge of δ_H 1.88 (ddd, *J*= 13.4, 4.6, 2.6 Hz; H-9a), 0.94 (overlap; H-9b), 2.12 (ddd, *J*= 13.4, 13.1, 4.6 Hz; H-10a), and 1.96 (ddd, *J*= 13.4, 4.6, 2.6 Hz; H-10b).



HMBC experiment was employed to connect both fragments described above through the correlations from δ_H 1.88 (H-9a) and 0.94 (H-9b) to δ_C 69.6 (C-8) and 56.9 (C-13), and from δ_H 2.12 (H-10a) and 1.96 (H-10b) to δ_C 69.6 (C-8), 150.2 (C-11), and 47.2 (C-12). Through the other direction, the correlation form δ_H 1.65 (H-1) to δ_C 150.2 (C-11) and 47.2 (C-12), also allowed the connection of fragments **a** to **b**. The vinyl protons (δ_H 4.79; H-20a and 4.70; H-20b), and their carbon counterpart (δ_C 106.5; C-20) was placed on C-11 by the correlation from both vinyl protons to δ_H 150.2 (C-11), 34.3 (C-10), and 47.2 (C-12). The isopropyl group, comprising two singlet methyls at δ_H 1.24 (δ_C 29.0; 16-CH_3) and δ_H 1.25 (δ_C 27.7; 17-CH_3), and a quaternary carbon at δ_C 53.6 (C-15), was place on C-14 due to the HMBC correlations from 16-CH_3 and 17-CH_3 protons to δ_C 45.4 (C-14).

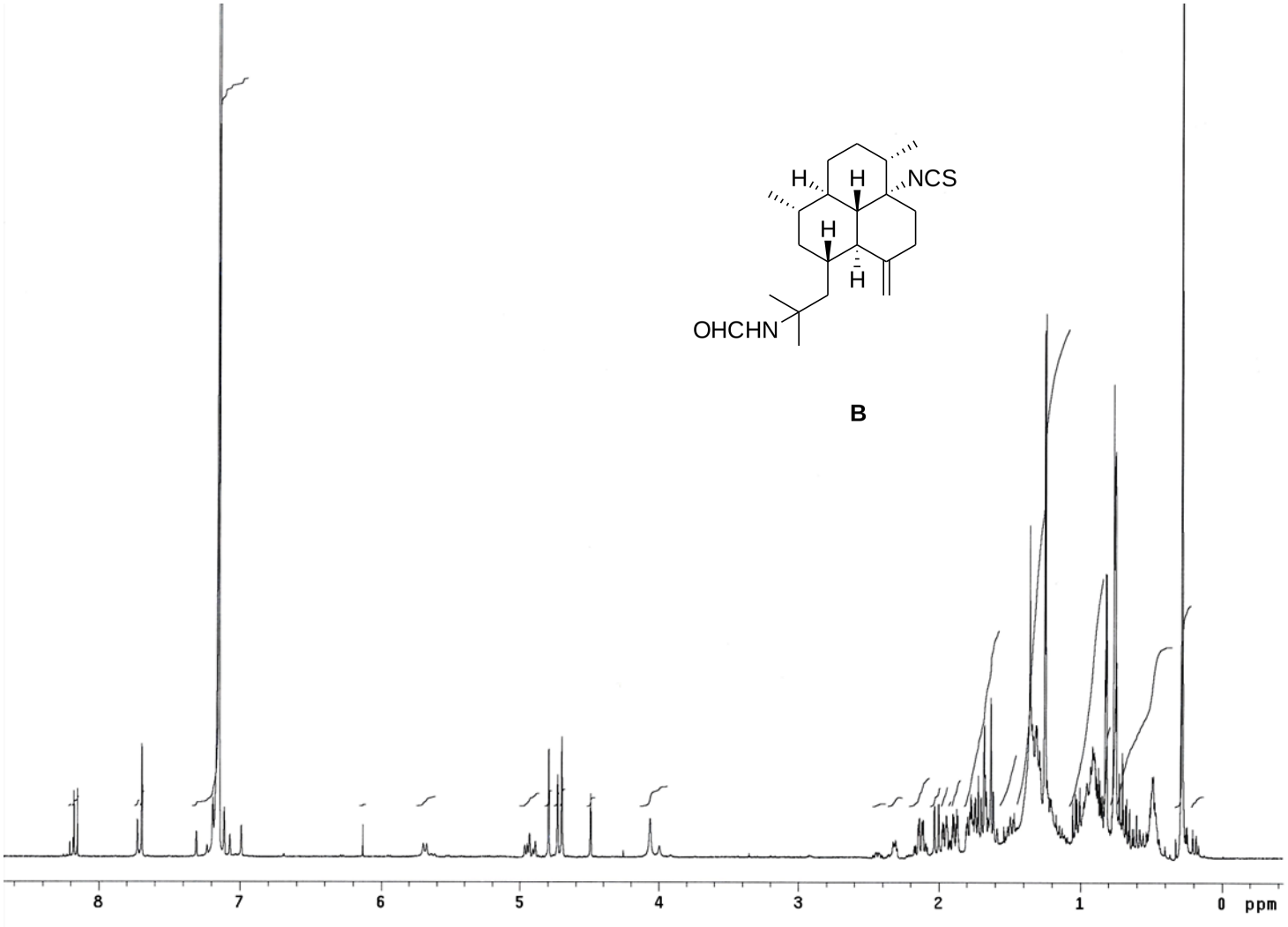


Figure 9. ^{1}H NMR spectrum of **B** (500 MHz, C_6D_6)

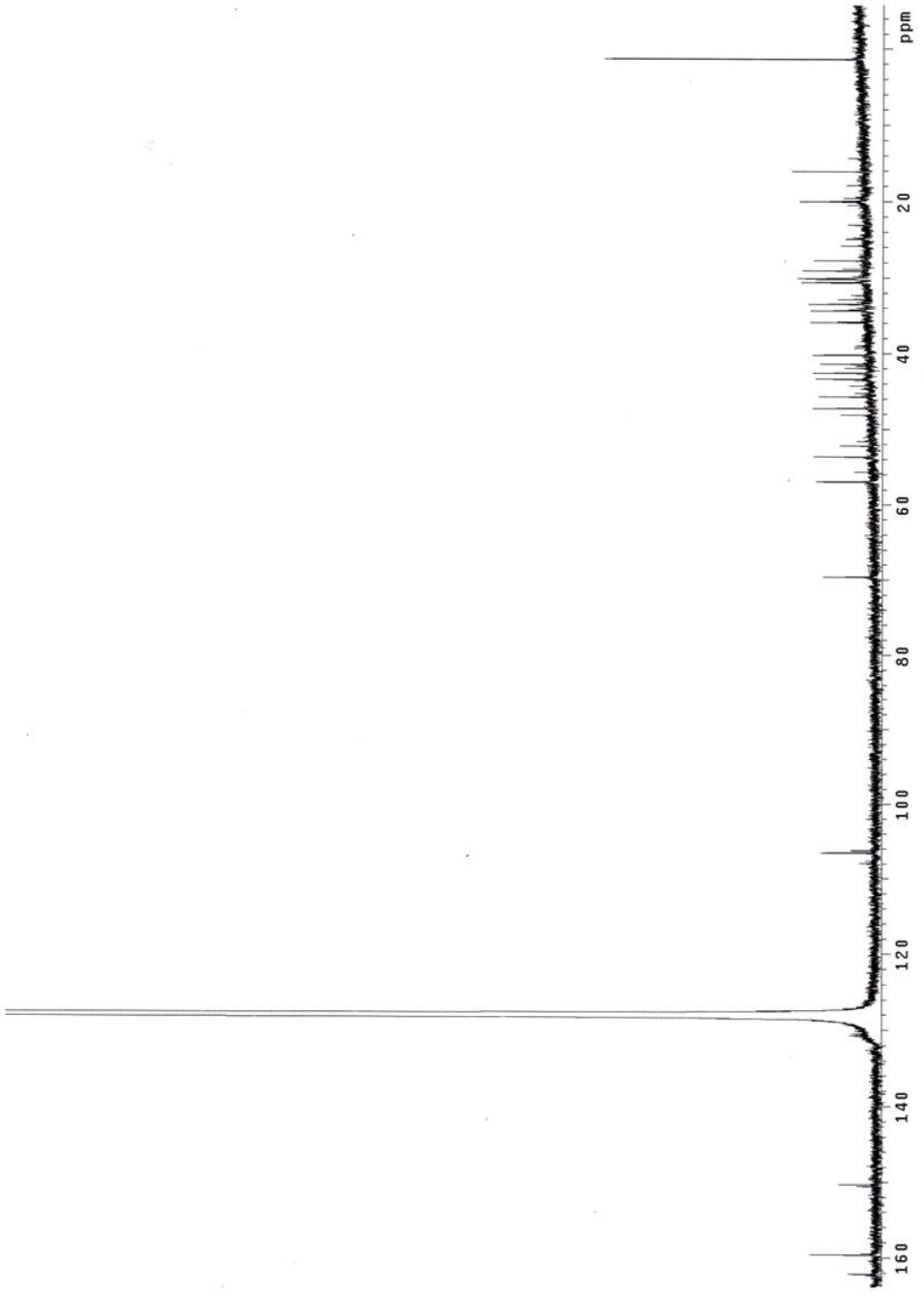
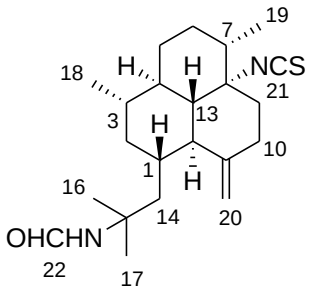


Figure 10. ^{13}C NMR spectrum of **B** (125 MHz, C_6D_6)

In the same manner as that for compound **A**, the formyl group was placed on C-15, as suggested by the chemical shift of the carbon bearing an amide group (δ_C 53.6; C-15), and the HMBC correlation from the formyl proton at δ_H 7.69 (H-22) to C-15. An isothiocyanate group was placed on C-8 as indicated by the characteristic chemical shift of the adjacent carbon (δ_C 69.6; C-8). The down-field shift from 65.7 ppm in compound **A** to 69.6 ppm in compound **B**, and the IR absorption band of ν 2075 cm^{-1} confirmed the presence of the isothiocyanate functionality. Compound **B** is therefore proposed here as a new isothiocyanate analog of **A**, named 8-isothiocyanato-15-formamidoamphilect-11(20)-ene. The relative configuration of **B** as shown 1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S** was drawn based on the assumption of the same organism source.



B

Table 3. NMR data of B (500 MHz for ^1H and 125 MHz for ^{13}C; C_6D_6)

position	^{13}C (mult)		^1H (*J* in Hz)	HMBC correlation (H⟶C)
1	33.4 (CH)	[32.8]	1.65, overlap	C-2, C-11, C-12, C-15
2a	41.3 (CH_2)	[41.9]	1.78, overlap	
b			0.62, ddd (14.6, 10.0, 3.4)	
3	35.9 (CH)	[35.8]	0.76, overlap	C-18
4	43.3 (CH)	[43.3]	0.94, overlap	C-5
5a	30.1 (CH_2)		1.75, overlap	C-13
b			0.48, m	

Table 3. (cont.)

position	^{13}C (mult)		^{1}H (*J* in Hz)	HMBC correlation (H→C)
6a	30.5 (CH_2)	[30.1]	1.31, br dd (13.4 3.9)	C-5
b			1.22, overlap	
7	42.5 (CH)	[42.5]	0.92, overlap	C-6, C-19
8	69.6 (C)		-	
9a	40.1 (CH_2)	[41.1]	1.88 (ddd; 13.4, 4.6, 2.6)	C-8, C-13
b			0.94 (overlap)	
10a	34.3 (CH_2)	[34.2]	2.12 (ddd; 13.4, 13.1 4.6)	C-8, C-9, C-11, C-12
b			1.96 (ddd; 13.4, 4.6, 2.6)	
11	150.2 (C)	[151.5]	-	
12	47.2 (CH)	[47.1]	1.69 (br d; 10.6)	C-11
13	56.9 (CH)		0.70 (br dd; 10.6, 10.0)	
14a	45.4 (CH_2)	[48.1]	2.02 (dd; 14.6, 1.7)	C-1, C-2, C-12, C-15
b			1.02 (dd; 14.6, 10.0)	C-16, C-17
15	53.6 (C)			
16	29.0 (CH_3)	[28.8]	1.24 (s)	C-14, C-15, C-17
17	27.7 (CH_3)	[30.6]	1.25 (s)	C-14, C-15, C-16
18	20.0 (CH_3)	[19.0]	0.76, d (5.8), [0.82, overlap]	C-2, C-3, C-4
19	16.0 (CH_3)	[14.3]	0.75, d (6.1), [0.82, overlap]	C-7, C-8
20a	106.5 (C)	[106.2]	4.79, s, [4.72, s]	C-10, C-11, C-12
b			4.70, s, [4.49, s]	
21-N*C*S	130.0 (C)			
22-NH*CH*O	159.6 (CH)	[162.1]	7.69, br d (1.7), [8.16, d, 12.1]	C-15
22-N*H*CHO			4.06, br s, [5.68, d, 12.1]	

Note; the chemical shifts of the minor conformer are presented in brackets.

3.1.2.3 Compound C

Compound **C** (4.8 mg, 0.67%) was obtained as a yellow oil. The molecular formula of **C** was proposed to be $C_{22}H_{34}N_2O$ from the pseudomolecular peak of $[M+Na]^+$ at *m/z* 365 in the ESI-mass spectrum. The unsaturation degree of 7 was deduced to be three rings, one exomethylene, one isonitrile, and one formamide. The IR spectrum of **C** showed the absorption bands at ν 3250 and 1670 cm^{-1}, belonging to the formamide moiety similar to those in compounds **A** and **B**. The bands of isocyanate (ν 2250 cm^{-1}) and isothiocyanate (ν 2075 cm^{-1}), shifted to ν 2130 cm^{-1}, and was identified to be an isonitrile group.

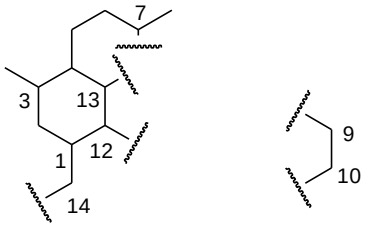
The ^1H and ^{13}C NMR spectra (Figures 11 and 12, Table 4) of **C** showed the two sets of signals of the rotameric mixture of *cisoid* and *transoid* in a ratio 3:2 similar to that of compounds **A** and **B**. In the same fashion as that for **A** and **B**, the discussion here is focusing on the major conformer, and the chemical shifts of the minor one are bracketed in Table 4.

The ^1H and ^{13}C NMR spectra of **C** were also almost identical to that of **A** and **B**. The ^{13}C NMR spectrum of compound **C** showed 22 carbon signals, classified to be four quaternary carbons, seven methines, seven methylenes, and four methyls as indicated by the DEPT experiments.

In the low-field region of the ^1H NMR spectrum, two spin systems were observed. The first one comprises the resonances at δ_H 7.70 (br d, *J*= 1.9 Hz; 22-NHC*H*O), and 4.12 (br s; 22-N*H*CHO), which are consistent to the formamide moiety as observed previously in compounds **A** and **B**. The other one is the resonances at δ_H 4.80 (s; H-20a) and 4.71 (s; H-20b), which are assigned for the exomethylene unit.

Connecting all the aliphatic methylene and methine signals were carried out by means of the COSY experiment in the same manner as that for compounds **A** and **B**. Fragment **a** is composed of a spin system of a six-membered alicyclic ring of δ_H 1.68 (overlap; H-1) connecting to 1.77 (overlap; H-2a), 0.66 (overlap; H-2b), 0.77 (overlap; H-3), 1.09 (br d, *J*= 10.9 Hz; H-4), 0.67 (overlap; H-13), and 1.86 (br dd, *J*= 11.2, 10.9 Hz; H-12). On C-4, an extension

toward δ_H 1.78 (overlap; H-5a), 0.51 (br dd, *J*= 12.6, 12.2 Hz; H-5b), 1.44 (m; H-6a), 1.22 (m; H-6b), 0.82 (overlap; H-7), and 0.75 (d, *J*= 5.8 Hz; H-19) was observed. To the other ends, on C-1 and C-3, the extensions were toward a methylene (δ_H 2.02, dd, *J*= 14.6, 1.7 Hz; H-14a and 1.04 (dd, *J*= 14.6, 10.4 Hz; H-14b), and a methyl (0.77, br s, H-18), respectively. As for fragment **b**, an ethylene bridge of 1.93 (br dd, *J*= 13.1, 1.9 Hz; H-9a), 0.84 (overlap; H-9b), 2.26 (ddd, *J*= 13.6 12.9, 1.9 Hz; H-10a), and 1.97 (overlap; H-10b) were detected.



a **b**

Also in the same fashion as that for compounds **A** and **B**, the connection of fragments **a** and **b** relied on the HMBC correlations. The long-range H-C correlation from δ_H 1.93 and 0.84 (H-9a and H-9b) to δ_C 55.6 (C-13) and 150.2 (C-11), from δ_H 2.26 and 1.97 (H-10a and H-10b) to δ_C 66.7 (C-8), 150.2 (C-11), and 46.6 (C-12), from δ_H 0.67 (H-13) to δ_C 150.2 (C-11), and from δ_H 4.80 and 4.71 (H-20a and H-20b) to δ_C 150.2 (C-11), allowed the connection of fragments **a** and **b** and also to the exomethylene moiety. The isopropyl group was placed on C-14 due to the correlation from δ_H 1.25 (16-CH_3 and 17-CH_3) to δ_C 45.5 (C-14).

The placement of the formamide group as described above on C-15 referred to the chemical shift at δ_C 53.6 (C-15). In addition to the characteristic IR absorption, the presence of the isonitrile group was confirmed by the chemical shift at 159.9 ppm, and the characteristic ^{13}C-^{14}N coupling (t, *J*= 4.2 Hz). This isonitrile group substituted at C-8 due to the chemical shift at 66.7 ppm of C-8.

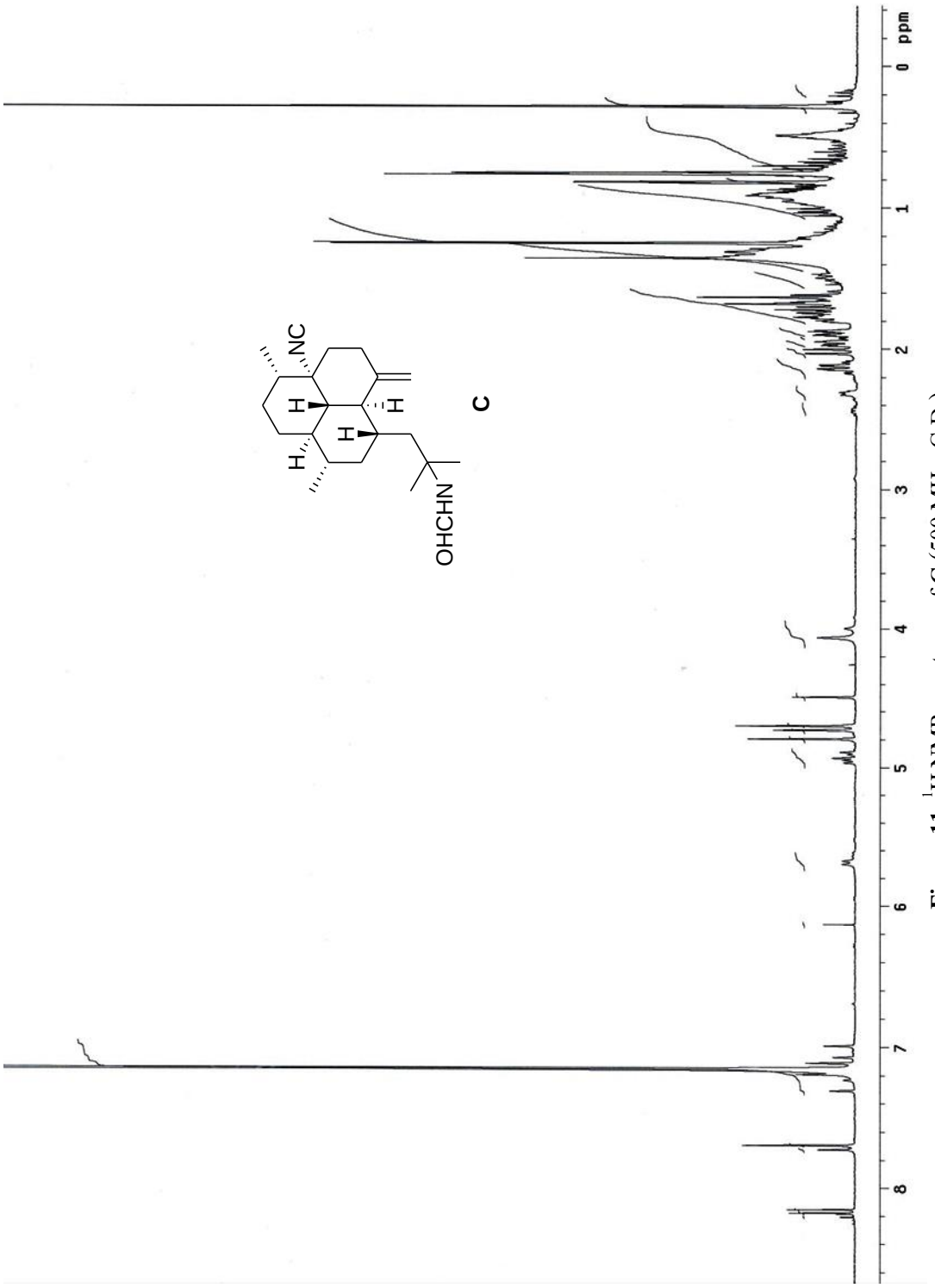


Figure 11. ^{1}H NMR spectrum of **C** (500 MHz, C_6D_6)

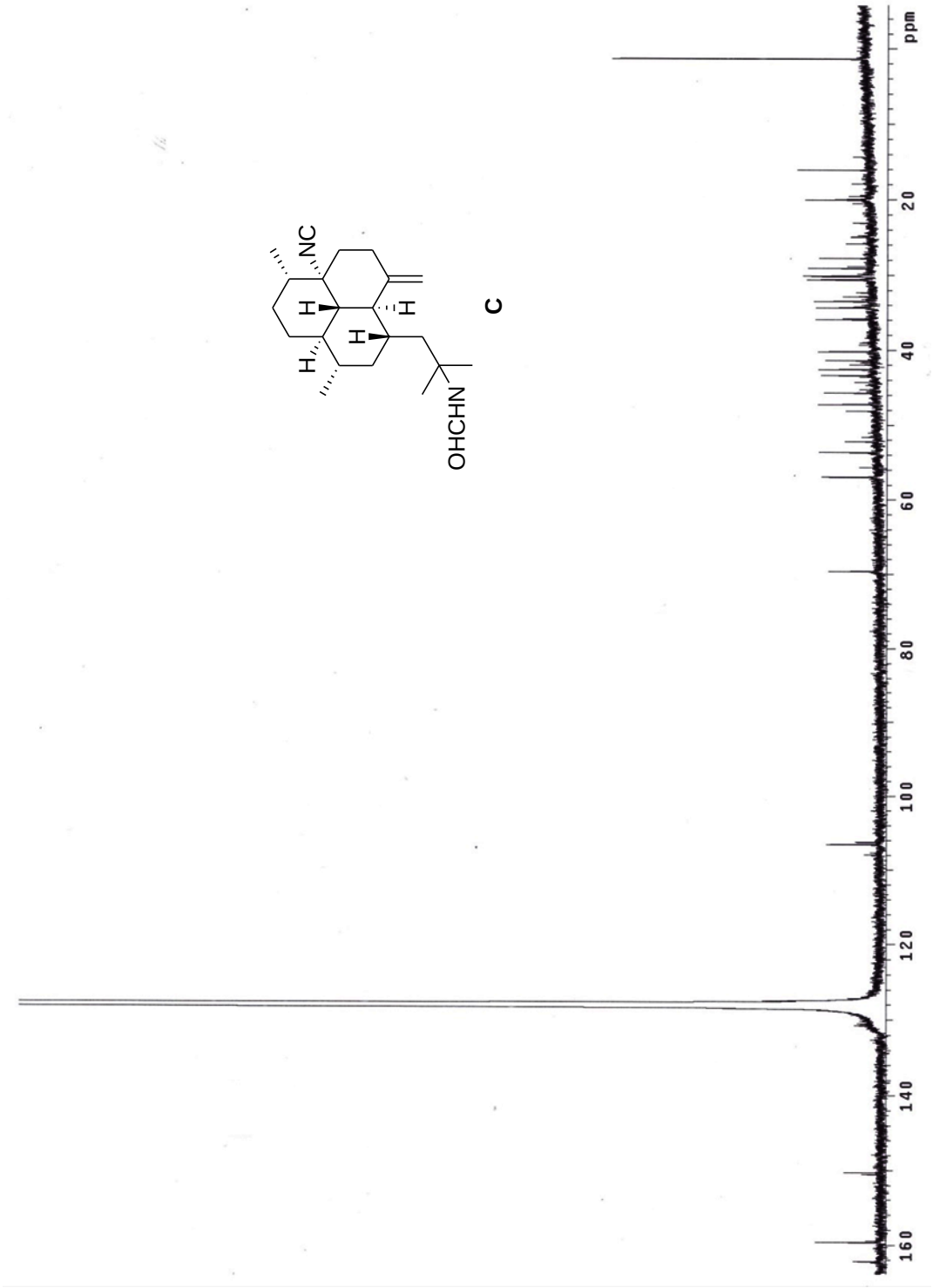
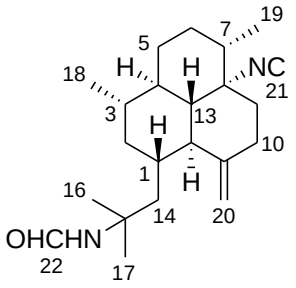


Figure 12. ^{13}C NMR spectrum of **C** (125 MHz, C_6D_6)

Compound **C** was therefore proposed to be 8-isocyano-15-formamidoamphilect-11(20)-ene (**153**). The compound was previously reported from the sponge *Hymeniacidon amphilecta* (Wratten and Faulkner, 1978) and *Svenzea flava* (Avilés et al, 2013). The configuration of **C** 1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S** as shown here refers to those of **A** and **B**, as indicated by the similarity in the NMR chemical shifts and coupling constants among **A**, **B**, and **C**. The specific rotation ($[\alpha]_D$= -22°; *c* 0.19, CH_2Cl_2) was comparable to that reported previously (lit. $[\alpha]_D$= -24°; *c* 1.0, $CHCl_3$; Wratten and Faulkner, 1978), therefore confirming the proposed configuration.



C

Table 4. NMR data of C (500 MHz for ^{1}H and 125 MHz for ^{13}C; C_6D_6)

position	^{13}C (mult)		^{1}H (*J* in Hz)	HMBC correlation (H⟶C)
1	33.3 (CH)	[32.6]	1.68, overlap	C-11, C-12, C-14
2a	41.2 (CH_2)	[41.7]	1.77, overlap	
b			0.66, overlap	
3	35.7 (CH)	[35.6]	0.77, overlap	C-2, C-4, C-18
4	42.7 (CH)	[42.7]	1.09, br d (10.9)	C-5, C-13
5a	30.0 (CH_2)	[29.9]	1.78, overlap	C-4, C-6
b			0.51, br dd (12.6, 12.2)	
6a	30.1 (CH_2)	[30.1]	1.44, m	
b			1.22, m	
7	40.8 (CH)	[40.8]	0.82, overlap	C-19
8	66.7 (C, t, *J*= 4.2 Hz)			

Table 4. (cont.)

position	^{13}C (mult)		^{1}H (*J* in Hz)	HMBC correlation (H→C)
9a	39.7 (CH_2)		1.93, br dd (13.1, 1.9)	C-11, C-13
b			0.84, overlap	
10a	33.8 (CH_2)	[33.8]	2.26, ddd (13.4, 12.9, 1.9)	C-8, C-9, C-11, C-12
b			1.97, overlap	
11	150.2 (C)	[151.4]		
12	46.6 (CH)	[46.5]	1.86, br dd (11.2, 10.9)	C-11
13	55.6 (CH)	[55.5]	0.67, overlap	C-11
14a	45.5 (CH_2)	[48.0]	2.02, dd (14.6, 1.7)	C-1, C-12, C-15,
b			1.04, dd (14.6, 10.4)	C-16, C-17
15	53.6 (C)	[52.2]		
16	29.1 (CH_3)	[28.0]	1.25, s, [0.86, s]	C-14, C-15, C-17
17	27.7 (CH_3)	[30.6]	1.25, s, [0.85, s]	C-14, C-15, C-16
18	20.0 (CH_3)	[19.0]	0.77, br s	C-2, C-3
19	16.0 (CH_3)	[14.3]	0.75, d (5.8)	C-7, C-8
20a	106.5 (C)	[106.2]	4.80, s, [4.74, s]	C-10, C-11, C-12
b			4.71, s, [4.52, s]	
21-N*C*	159.9 (C, t, *J*= 4.2 Hz)			
22-NH*CH*O	159.6 (CH)	[162.4]	7.70, br d (1.9), [8.17, d, 11.9]	C-15
22-N*H*CHO			4.12, br s, [6.09, d, 11.9]	

Note; the chemical shifts of the minor conformer are presented in brackets.

3.1.2.4 Compound D

Compound **D** (3.8 mg, 0.53%) was obtained as a yellow oil. The molecular formula was proposed as $C_{21}H_{33}NO$ from the pseudomolecular mass of $[M+H]^+$ at *m/z* 316 in the ESI-mass spectrum. The unsaturation degree of 6 was deduced to be three rings, two double bonds, and one formamide.

Similar to compounds **A**, **B** and **C**, the ^{1}H and ^{13}C NMR signals in the ratio of 3:2 were also observed in the ^{1}H and ^{13}C NMR spectra of compound **D**. In the same manner as that for the previous three compounds, the major signals were described here, and the chemical shifts of the minor conformer are bracketed in Table 5. The ^{13}C NMR spectrum of compound **D** showed the signals of 21 carbons and were identified as four quaternary carbons, eight methylenes, six methines, and three methyls. Notice that, whereas the formamide moiety can be observed in **D** as indicated by the IR absorption bands at ν 3250 and 1670 cm^{-1}, and by the ^{13}C resonances at δ_C 159.4 (major; C-21) and δ_C 162.1, (minor; C-21), compound **D** did not showed the presence of secondary functional groups as previously observed in compounds **A**, **B**, or **C**.

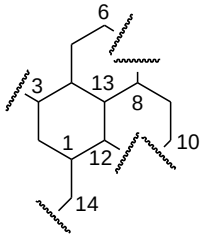
The ^{1}H NMR spectrum showed the resonances at δ_H 7.69 (d, *J*= 1.7 Hz; H-22) and 8.16, d, *J*= 12.1 Hz; H-22) identified as a formamide moiety, and at δ_H 4.93 (s; H-20a), 4.70 (s; H-20b), 4.87 (s; H-16a), and 4.82 (s; H-16b) for an exomethylene and a terminal vinyl groups.

The remaining signals belong to an extended aliphatic spin system which was deduced from the COSY experiment. Starting from a methylene at δ_H 2.26 (br dd, *J*= 9.2, 6.1 Hz; H-10), the correlations allowed the connection through δ_H 1.36 (overlap; H-9a) and 1.16 (m; H-9b), 2.09 (dd, *J*= 10.9, 9.0 Hz; H-8), 1.05 (br d, *J*= 9.0 Hz; H-13), 1.33 (overlap; H-12), 1.71 (br dd, *J*= 11.2, 3.6 Hz; H-1), 1.96 (ddd, *J*= 13.4, 4.1, 3.9 Hz; H-2a), 0.60 (overlap; H-2b), 0.91 (m; H-3), 0.63 (br dd, *J*= 13.6, 11.4 Hz; H-4), 1.32 (overlap; H-5a), 1.34 (overlap; H-5b), and finally to δ_H 1.34 (m; H-6a), and 1.00 (overlap; H-6b).

An isobutylene moiety was placed on C-1, based on the COSY correlation between H-1 (δ_H 1.71, br dd, *J*= 11.2, 3.6 Hz) and H-14 (δ_H 2.80, br d, *J*= 13.6 Hz and 1.51, dd, *J*= 13.6, 10.4 Hz), and on the HMBC correlation from δ_H 4.87 (s; H-16a) and 4.82 (s: H-16b) to δ_C 144.5 (C-15) and 42.8 (C-14). Two methyl groups, H-18 and H-19, were placed on C-3 and C-7, respectively, according to the correlations from δ_H 0.82 (d, *J*= 6.3 Hz; H-18) to δ_C 42.4 (C-2) and 39.2 (C-3), and from δ_H 1.34 (s; H-19) to δ_C 55.5 (C-7) and 41.5 (C-8).

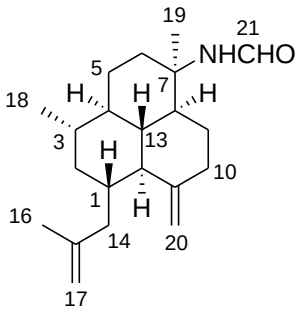
The exomethylene of C-11 and C-20 connects between C-10 and C-12 due to the correlations from δ_H 4.93 (s; H-20a) and 4.70 (s; H-20b) to δ_C 34.8 (C-10) and 51.3 (C-12).

As for the formamide moiety, unlike compounds **A**, **B**, and **C**, the amine group substituted on C-7, as indicated by the chemical shift of C-7, at δ_C 55.5. The structure of compound **D** was therefore identified to be 7-formamidoamphilecta-11(20),15-diene.



Compared with the chemical shift of (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-7-formamidoamphilecta-11(20),15-diene reported from the sponge *Cymbastela hooperi* by Wright and Lang-Unnasch (Wright and Lang-Unnasch, 2009), however, the chemical shift of C-19 assigned here (δ_C 24.8) as compared with that reported by Wright and Lang-Unnasch (δ_C 20.3) (Wright and Lang-Unnasch, 2009), suggested the different orientation. Compound **D** was proposed as a 7-epimer of (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-7-formamidoamphilecta-11(20),15-diene.

The relative configuration depicted here refers to those of compounds **A**, **B**, and **C**, presumably based on the same producing species hypothesis.



D

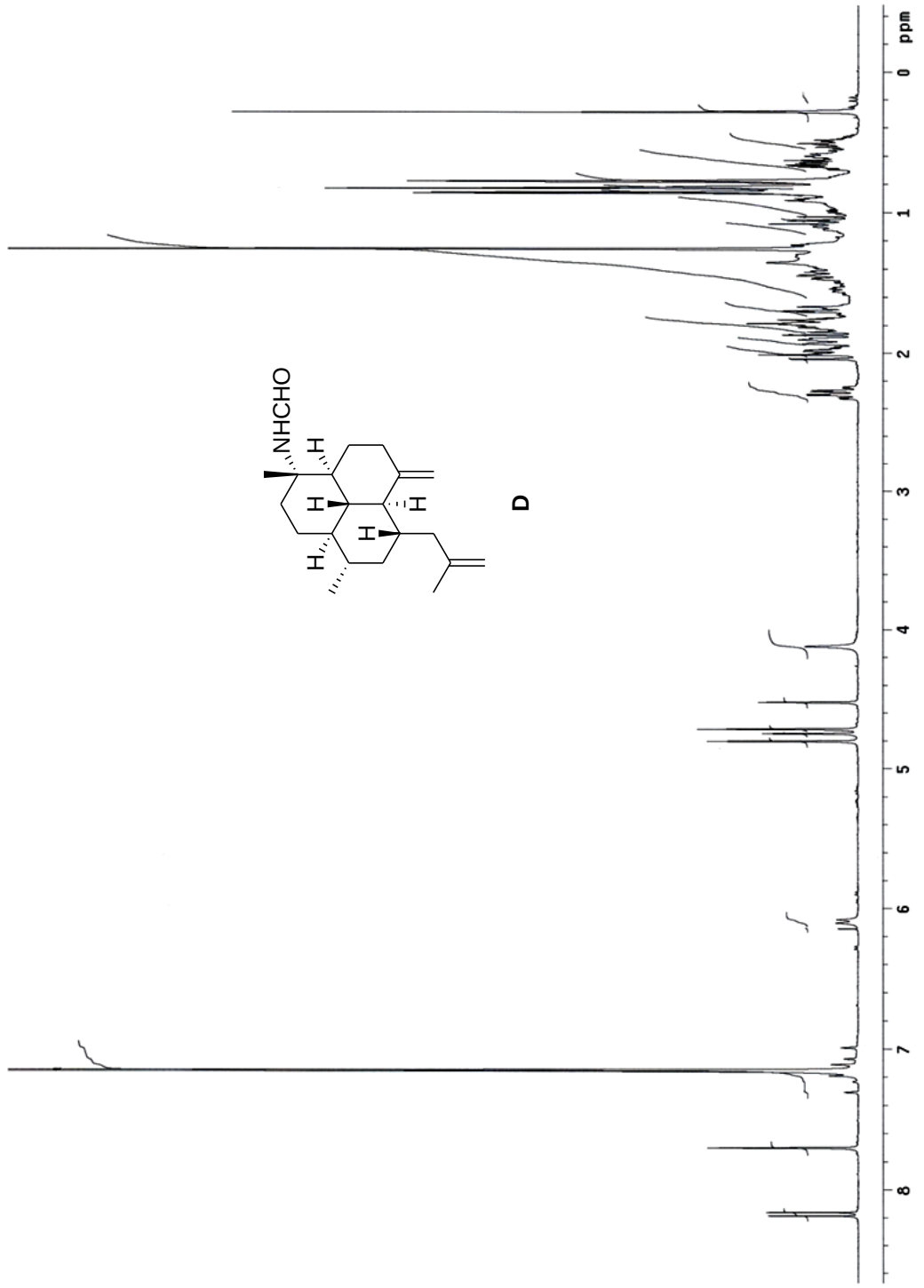
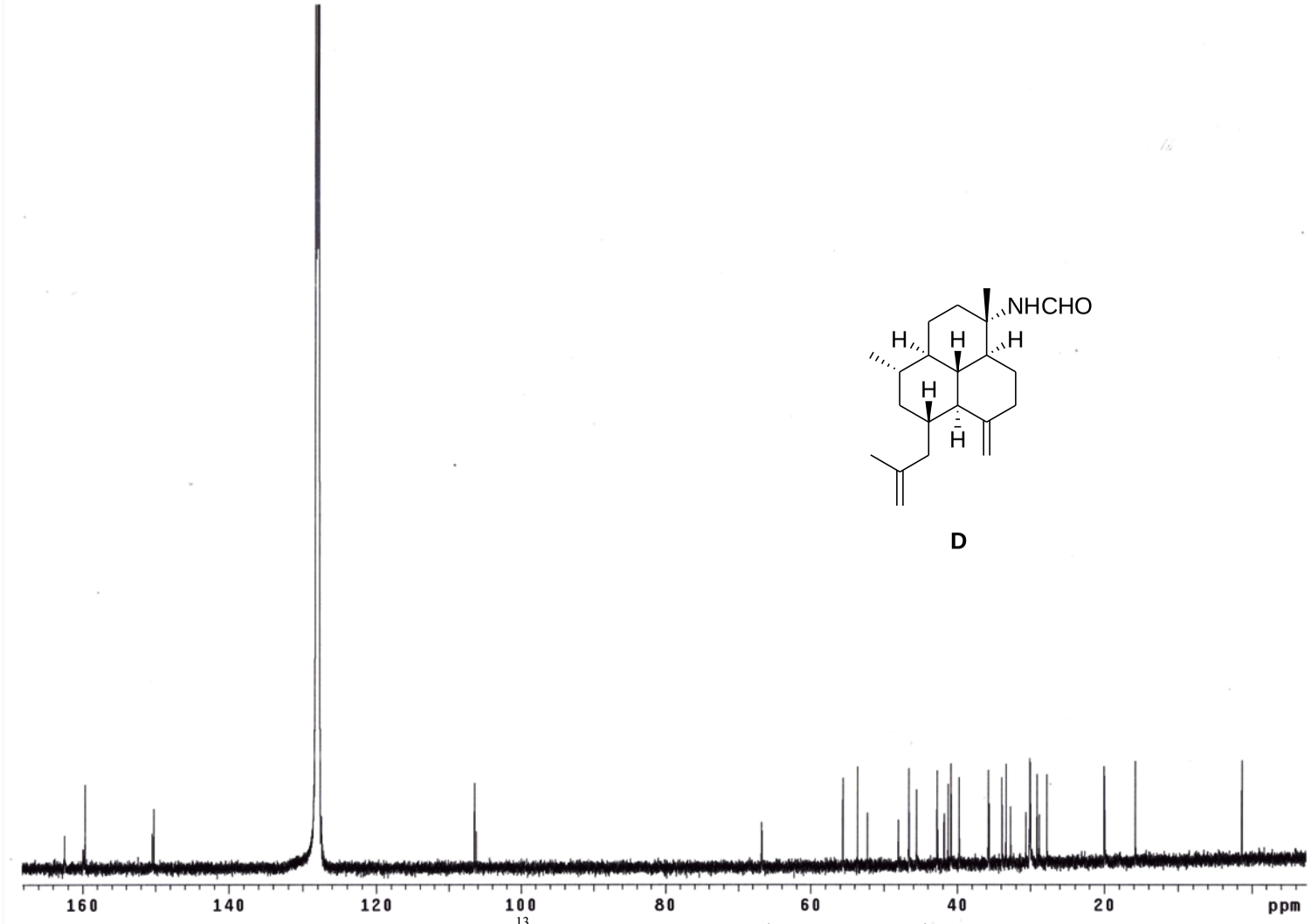


Figure 13. ^{1}H NMR spectrum of **D** (500 MHz, C_6D_6)



D

Figure 14. ^{13}C NMR spectrum of **D** (125 MHz, C_6D_6)

Table 5. NMR data of D (500 MHz for ^{1}H and 125 MHz for ^{13}C; C_6D_6)

position	^{13}C (mult)		^{1}H (*J* in Hz)	HMBC correlation (H→C)
1	35.4 (CH)	[32.8]	1.71, br dd (11.2, 3.6)	
2a	42.4 (CH_2)	[41.9]	1.96, ddd (13.4, 4.1, 3.9),	
			[1.88, ddd, 13.4, 4.1, 3.9]	
b			0.60, overlap	
3	39.2 (CH)	[39.4]	0.91, m	
4	44.0 (CH)	[44.4]	0.63, br dd (13.6, 11.4)	
5a	30.1 (CH_2)		1.32, overlap	C-4, C-7, C-13
b			1.34, overlap	
6a	33.5 (CH_2)	[33.2]	1.34, m	C-4, C-5, C-7
b			1.00, overlap	
7	55.5 (C)	[53.7]		
8	41.5 (CH)		2.09, dd (10.9, 9.0)	C-6, C-7, C-13
9a	20.7 (CH_2)	[20.3]	1.36, overlap	C-7, C-11, C-20
b			1.16, m	
10a	34.8 (CH_2)	[34.4]	2.26, br dd (9.2, 6.1)	C-11
b			2.26, br dd (9.2, 6.1)	
11	148.2 (C)	[148.4]		
12	51.3 (CH)	[50.6]	1.33, overlap	
13	46.0 (CH)	[46.2]	1.05, br d (9.0)	C-20
14a	42.8 (CH_2)	[42.6]	2.80, br d (13.6),	C-2, C-15, C-16
			[2.75, br d, 13.6]	
b			1.51, dd (13.6, 10.4)	
15	144.5 (C)			
16a	111.6 (CH_2)	[111.7]	4.87, s, [4.93, s]	C-14, C-15
b			4.82, s [4.82, s]	
17	22.5 (CH_3)	[30.7]	1.69, s	C-14, C-15, C-16

Table 5. (cont.)

position	^{13}C (mult)		^{1}H (*J* in Hz)	HMBC correlation (H→C)
18	19.6 (CH_3)	[19.5]	0.82, d (6.3), [0.74, d, 6.3)	C-2, C-3
19	24.8 (CH_3)	[24.6]	1.34, s	C-7, C-8
20a	107.1 (C)	[106.7]	4.93, s, [4.87, s]	C-10, C-12
b			4.70, s, [4.67, s]	
21-NH*CH*O	159.4 (CH)	162.1	7.72, br d (1.7), [8.15, d, 11.9]	
21-N*H*CHO			3.99, br s, [5.41, d, 11.9]	

Note; the chemical shifts of the minor conformer are presented in brackets.

3.2 Isolation and structure determination of compounds from the soft coral *Eleutherobia* sp.

3.2.1 Isolation and purification

The MeOH-extract of the soft coral *Eleutherobia* sp. was sub-fractionated to yield, hexane-, CCl_4-, $CHCl_3$-, *n*-BuOH- and H_2O-crude extracts (1.4 g, 724.0 mg, 458.1 mg, 270.0, and 282.2 mg, respectively). The $CHCl_3$-extract was chromatographed over a Sephadex LH-20 (MeOH) column to yield three major fractions. The first fraction was fractionated over SiO_2 HPLC (hexane/*i*-PrOH 87:13, 3.0 mL/min) and RP-C18 HPLC (MeCN/H_2O 1:3, 1.2 mL/min) columns to yield **E** (1.0 mg). The second fraction was separated with RP-C8 HPLC (MeCN/H_2O 1:19, 5.0 mL/min) to yield **F** (2.7 mg) and **G** (9.3 mg). Compound **F** (2.4 mg) was also obtained from the third fraction through SiO_2 HPLC (hexane/*i*-PrOH 4:1, 1.0 mL/min).

3.2.2 Structure determination

3.2.2.1 Compound E

Compound **E** (2.4 mg, 0.21% yield) was obtained as a white solid. The molecular formula of compound **E** was $C_{20}H_{28}O_4$ as indicated by the pseudomolecular peak of $[M+Na]^+$ at *m/z* 355 in the ESI-mass spectra. The unsaturation degree of 7 belongs to two rings,

four olefins, and one carbonyl. Twenty carbons were observed in the ^{13}C NMR spectrum, among which are seven methines, five methylenes, three methyls, and five quaternary carbons. The presences of hydroxyl and lactone carbonyl groups were indicated by the IR absorption bands at ν 3400 cm^{-1} and 1720 cm^{-1}, respectively.

Four olefinic groups were observed in the NMR spectra of **E** (Figures 15 and 16, Table 6). Among these, two were conjugated diene [δ_C 132.1 (C-4), 137.2 (C-12), 119.2 (C-13), 150.5 (C-14); δ_H 7.18 (overlap; H-12), 6.51 (dd, *J*= 15.5, 12.0 Hz; H-13), and 5.73 (d, *J*= 15.5; H-14)]. The coupling constants of H-13 (dd, *J*= 15.5, 12.0 Hz) and H-14 (d, *J*= 15.5; H-14) indicated a *trans* geometry. The chemical shifts of the four carbons suggested the mesomeric effect, hence the connection to the carbonyl carbon at δ_C 169.3 (C-3). This is supported by the HMBC correlation from δ_H 7.18 (H-12) to δ_C 119.2 (C-3).

The olefinic proton at δ_H 5.13 (d, *J*= 8.0 Hz; H-8) was a part of a trisubstituted olefin (δ_C 134.5; C-7, and 131.6; C-8). The COSY experiment extended this olefin to connect to a secondary alcohol of H-9 (δ_H 4.33, ddd, *J*= 10.0, 8.0, 5.5 Hz), then to H-10 (δ_H 2.37, dd, *J*= 13.5, 5.5 Hz and δ_H 1.97, dd, *J*= 13.5, 10.0 Hz). The HMBC correlations from δ_H 4.65 (H-19a) and 4.57 (H-19b) to δ_C 149.7 (C-11) and vice versa, and from both H-19 to δ_C 41.4 (C-11a) extended the spin system over an exomethylene (δ_H 4.57, s, H-19a, and 4.65, s, H-19b), then to a series of methylenes and methines of H-1 (δ_H 3.67, dd, *J*= 10.9, 5.6 Hz and 3.39, dd, *J*= 11.6, 10.9 Hz), H-11a (δ_H 2.01, ddd, *J*= 11.6, 6.6, 5.6 Hz), H-4a (δ_H 2.96, ddd, *J*= 12.0, 6.5, 5.5 Hz), H-5 (δ_H 1.60, overlap and δ_H 1.22, dddd, *J*= 13.0, 12.0, 5.5, 2.0 Hz), and H-6 (δ_H 2.15, ddd, *J*= 13.0, 13.0, 5.5 Hz and δ_H 1.46, ddd, *J*= 13.0, 5.5, 2.0 Hz).

With the HMBC correlations from δ_H 7.18 (H-12) to δ_C 39.9 (C-4a) and from δ_H 2.15 (H-6a), and 1.46 (H-6b) to δ_C 134.5 (C-7) and 22.6 (C-18), deduced an oxa-bicyclotridecane skeleton. A hydroxyl isopropyl terminal was placed on C-14. The structure of therefore is proposed to be xeniolide A. The compound was first reported from the soft coral *Xenia macrospiculata* (Kashman and Groweiss, 1978).

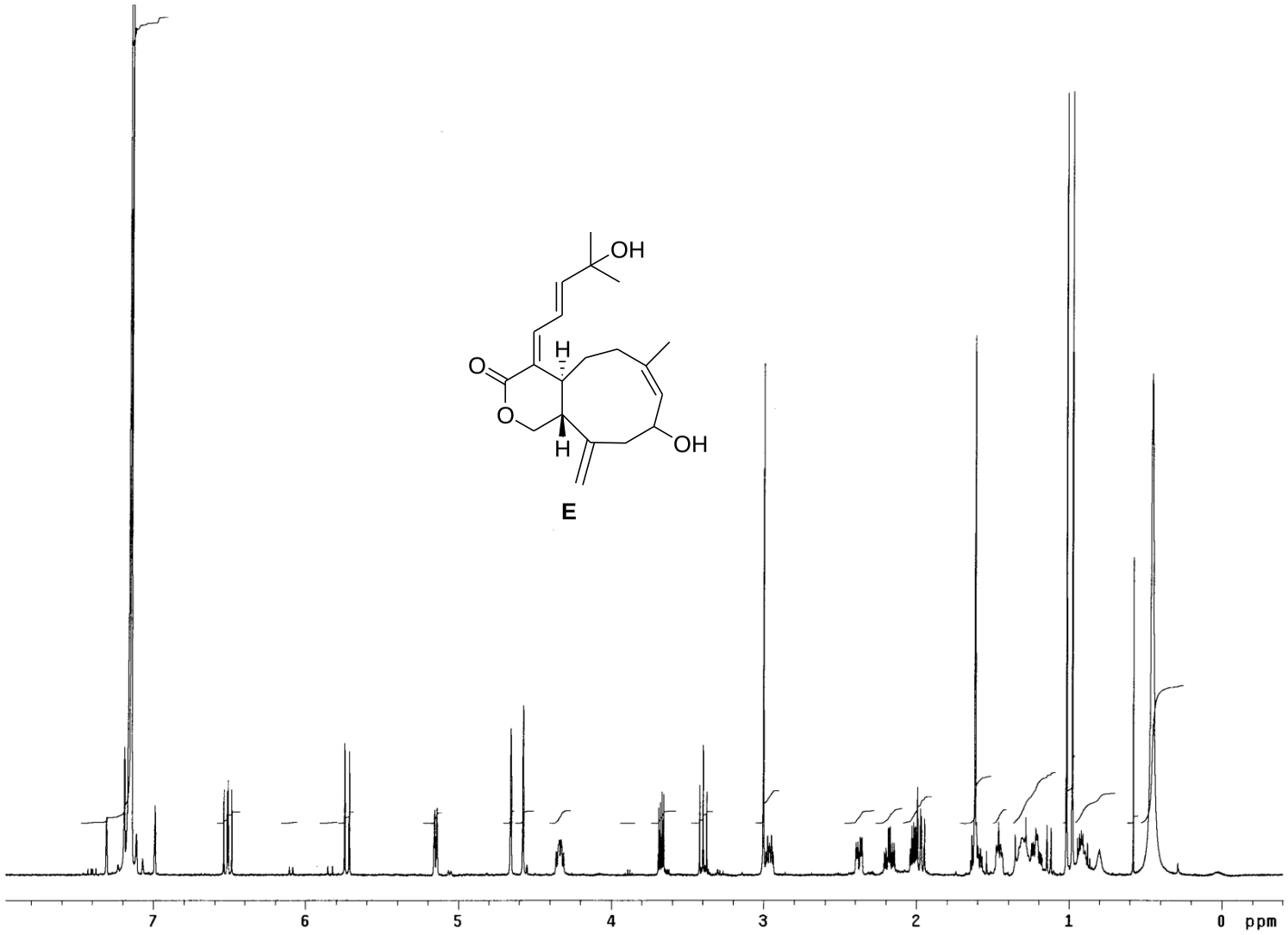


Figure 15. ^{1}H NMR spectrum of **E** (500 MHz, C_6D_6)

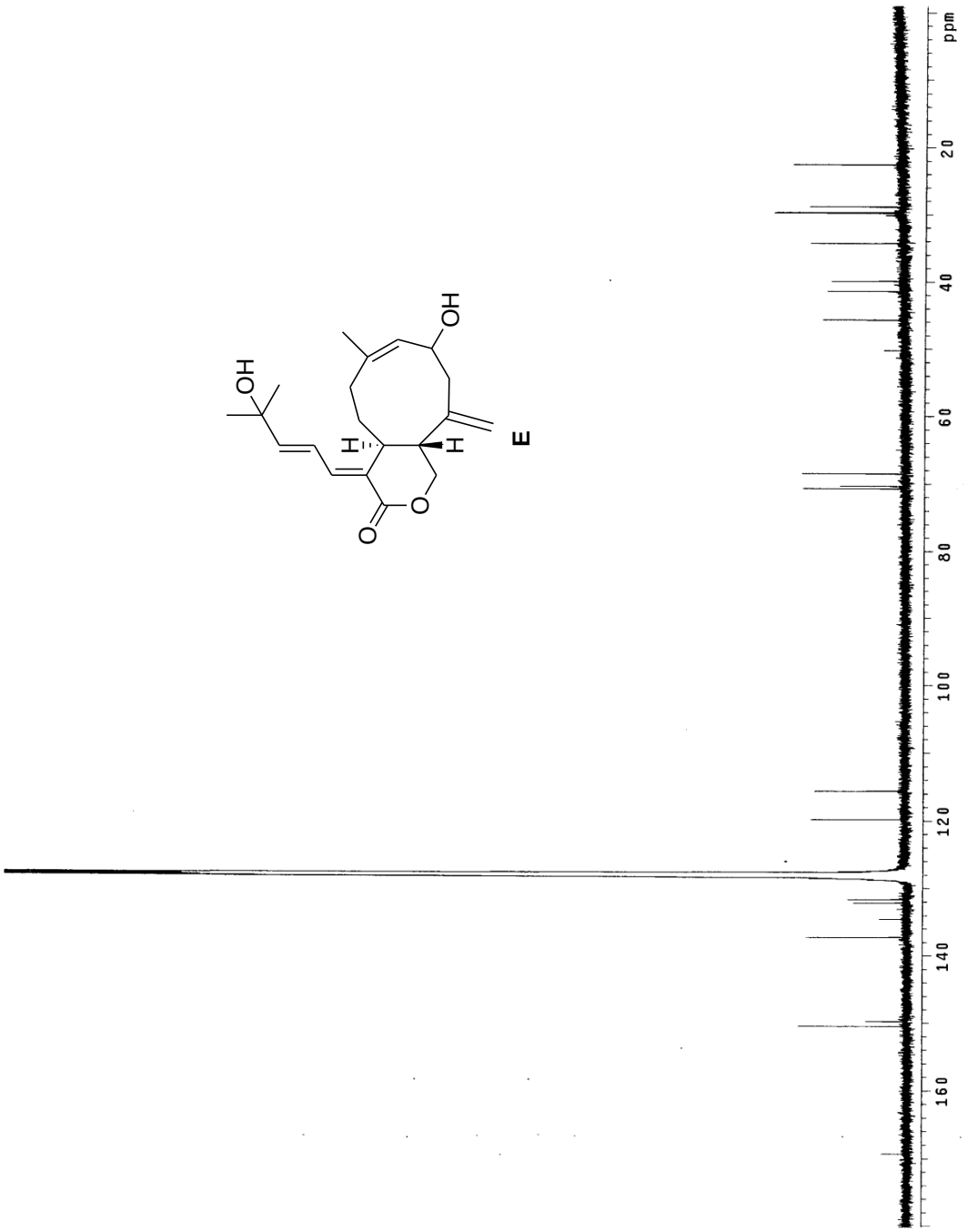
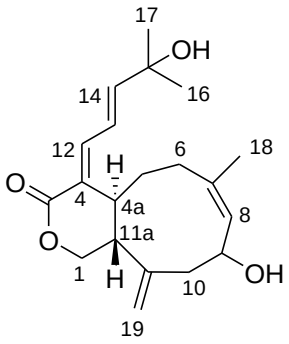


Figure 16. ^{13}C NMR spectrum of **E** (125 MHz, C_6D_6)



E

Table 6. NMR data of E (500 MHz for ^{1}H and 125 MHz for ^{13}C; C_6D_6)

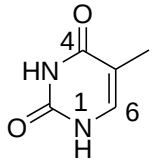
Position	^{13}C (mult)	^{1}H (*J* in Hz)	HMBC correlation (H→C)
1a	70.7 (CH_2)	3.67, dd (10.9, 5.6)	C-11, C-11a
b		3.39, dd (11.6, 10.9)	
3	169.3 (C)		
4	132.1 (C)		
4a	39.9 (CH)	2.96, ddd (12.0, 6.5, 5.5)	C-1, C-3, C-4, C-5, C-11, C-12
5a	34.3 (CH_2)	1.60, overlap	C-7
b		1.22, dddd (13.0, 12.0, 5.5, 2.0)	
6a	28.8 (CH_2)	2.15, ddd (13.0, 13.0, 5.5)	C-5, C-7, C-8, C-18
b		1.46, ddd (13.0, 5.5, 2.0)	
7	134.5 (C)		
8	131.6 (CH)	5.13, d (8.0)	C-6, C-18
9	68.5 (CH)	4.33, ddd (10.0, 8.0, 5.5)	C-7
10a	45.7 (CH_2)	2.37, dd (13.5, 5.5)	C-8, C-9, C-11, C-19
b		1.97, dd (13.5, 10.0)	
11	149.7 (C)		
11a	41.4 (CH)	2.01, ddd (11.6, 6.6, 5.6)	C-1, C-4, C-5, C-19
12	137.2 (CH)	7.18, overlap	C-3, C-4, C-4a, C-14
13	119.2 (CH)	6.51, dd (15.5, 12.0)	C-4, C-14, C-16
14	150.5 (CH)	5.73, d (15.5)	C-15, C-16, C-17

Table 6. cont.

Position	^{13}C (mult)	^{1}H (*J* in Hz)	HMBC correlation (H→C)
15	70.3 (C)	-	
16	29.7 (CH_3)	1.01, s	C-14, C-15, C-17
17	29.8 (CH_3)	0.97, s	C-14, C-15, C-16
18	22.6 (CH_3)	1.61, s	C-6, C-7, C-8
19a	115.5 (CH_2)	4.57, s	C-10, C-11
b		4.65, s	

3.2.2.2 Compound F

The molecular formula of compound **F**, which was isolated as a white solid (5.1 mg, 1.07% yield), was $C_5H_6N_2O_2$, as deduced from the $[M+Na]^+$ peak at *m/z* 149.0331 in HR-ESIMS spectrum (calcd. for $C_5H_6N_2O_2Na$ 149.0327). Among the five carbons, the ^{13}C NMR spectrum (Figure 18) indicated that two were carbonyls, two were an olefinic, and one was methyl. As for the ^{1}H, the NMR spectrum (Figure 17) showed only four singlet signals, two of which were exchangeable (δ_H 10.96; H-4, and 10.50; H-1), whereas the other two were one olefinic (δ_H 7.23) and one methyl (δ_H 1.71), corresponding well with the carbon resonances. The characteristic chemical shifts of the amide (δ_C 165.0; C-4) and imide (δ_C 151.6; C-2), and the mesomeric effect casting on C-5 (δ_C 107.7) and C-6 (δ_C 137.8), allowed the structure of compound **F** to be proposed as thymine. The chemical shifts of compound **F** were confirmed by direct comparison with those previously reported (Quiao and Uy, 2013).



F

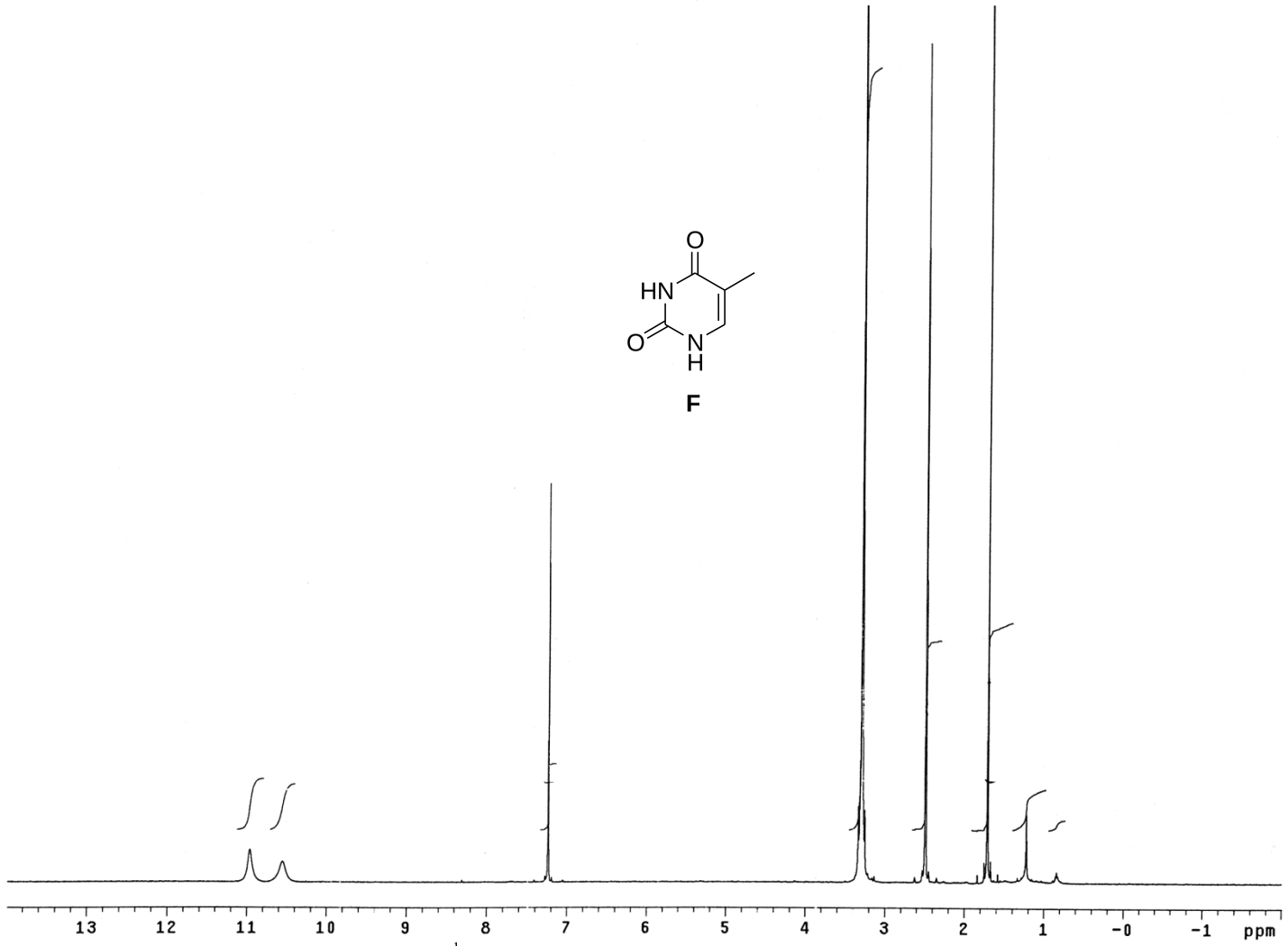


Figure 17. ^{1}H NMR spectrum of **F** (500 MHz, DMSO-d_6)

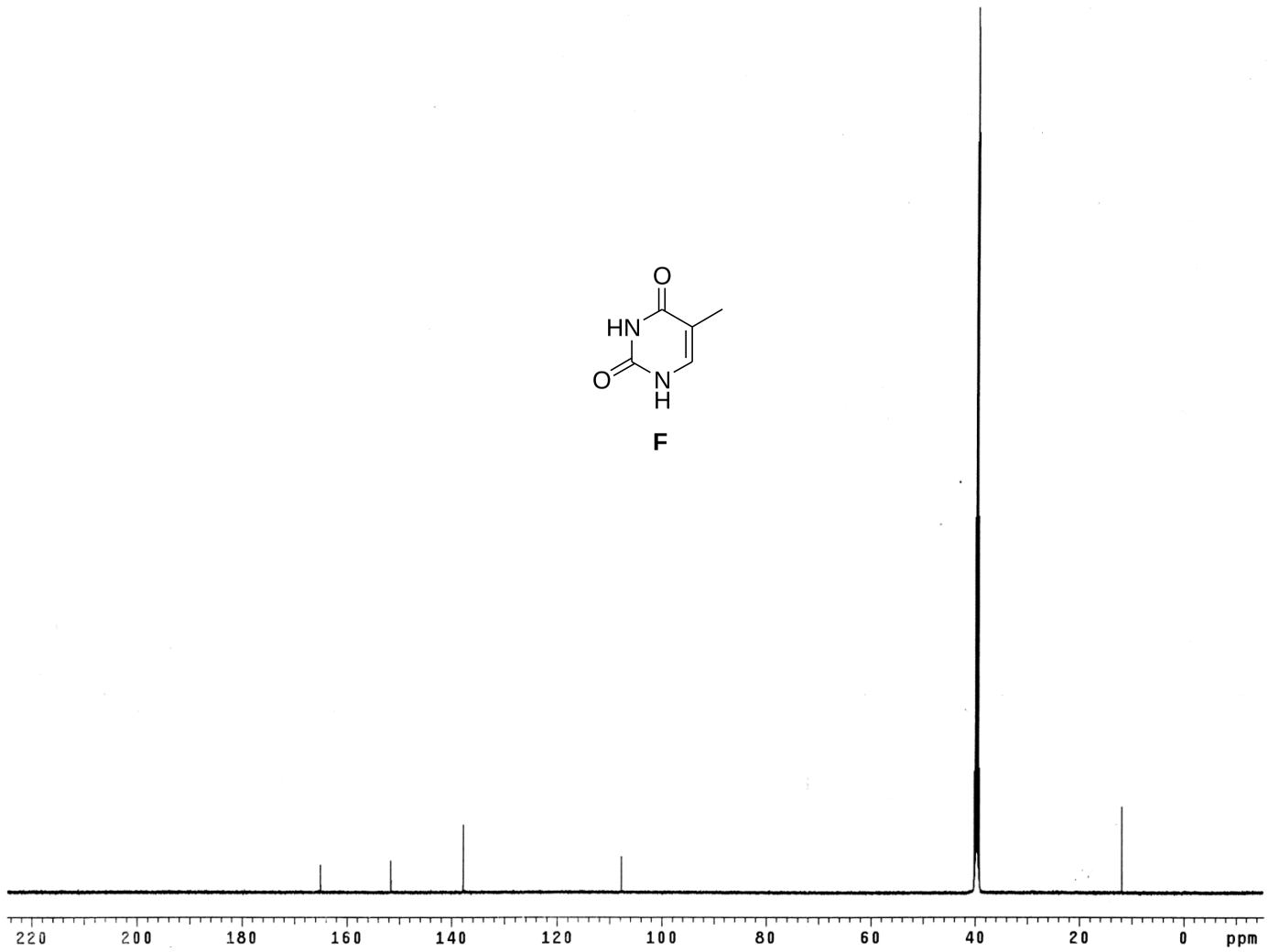


Figure 18. ^{13}C NMR spectrum of **F** (125 MHz, DMSO-d_6)

Table 7. NMR data of F (500 MHz for ^{1}H and 125 MHz for ^{13}C; DMSO-d_6)

position	^{13}C (mult)	^{1}H (mult; *J* in Hz)	HMBC correlation (H→C)
1 NH		10.50 (br s)	
2	151.6 (C)		
3 NH		10.96 (br s)	
4 N	165.0 (C)		
5	107.7 (C)		
6	137.8 (CH)	7.23, s	C-2, C-4, C-5
7	11.9 (CH_3)	1.71, s	C-4, C-5, C-6

3.2.2.3 Compound G

Compound **G** was isolated as a white solid (2.7 mg, 0.58% yield). The HRESIMS spectrum showed a $[M+Na]^+$ peak at *m/z* 193.0596, indicating the molecular formula of $C_7H_{10}N_2O_3$ (calcd. for $C_7H_{10}N_2O_3Na$ 193.0589). The unsaturation degree of 4 was deduced to two rings and two carbonyls. The ^{13}C NMR spectrum (Figure 20, Table 8) showed the signals of seven carbons, among which were four methylenes and three quaternary carbons. The presence of a hydroxyl group, an imide, and an amide carbonyl were observed at ν 3340 cm^{-1}, 1776 cm^{-1} and 1720 cm^{-1}, in the IR spectrum, respectively.

The ^{1}H NMR spectrum of compound **G** (Figure 19, Table 8) showed a spin system of four consecutive methylenes (δ_H 3.73, br dd, *J*= 13.1, 5.2 Hz, H-5a; 2.83, ddd, *J*= 12.9, 12.9, 3.4 Hz, H-5b; 1.62, overlap, H-6a; 1.26, dddd, *J*= 13.4, 13.4, 4.8, 4.8 Hz, H-6b; 1.63, overlap, H-7a; 1.70, dddd, *J*= 13.4, 13.4, 4.8, 4.8 Hz, H-7b; 1.83, br d, *J*= 13.1, 13.1, 4.1 Hz, H-8a; and 1.36, ddd, *J*= 13.1, 13.1, 4.1 Hz, H-8b).

Connecting the resulting butyrene bridge to three other quaternary carbons was achieved by means of HMBC correlation from δ_H 3.73 (H-5a) and 2.83 (H-5b) to δ_C 153.1 (C-3), 24.4 (C-6), 24.4 (C-7), and 82.1 (C-9), from δ_H 1.62 (H-6a) and 1.26 (H-6b) to δ_C 24.4 (C-7) and 31.5 (C-8), from δ_H 1.63 (H-7a) and 1.70 (H-7b) to δ_C 24.4 (C-6) and 35.5 (C-8), and from

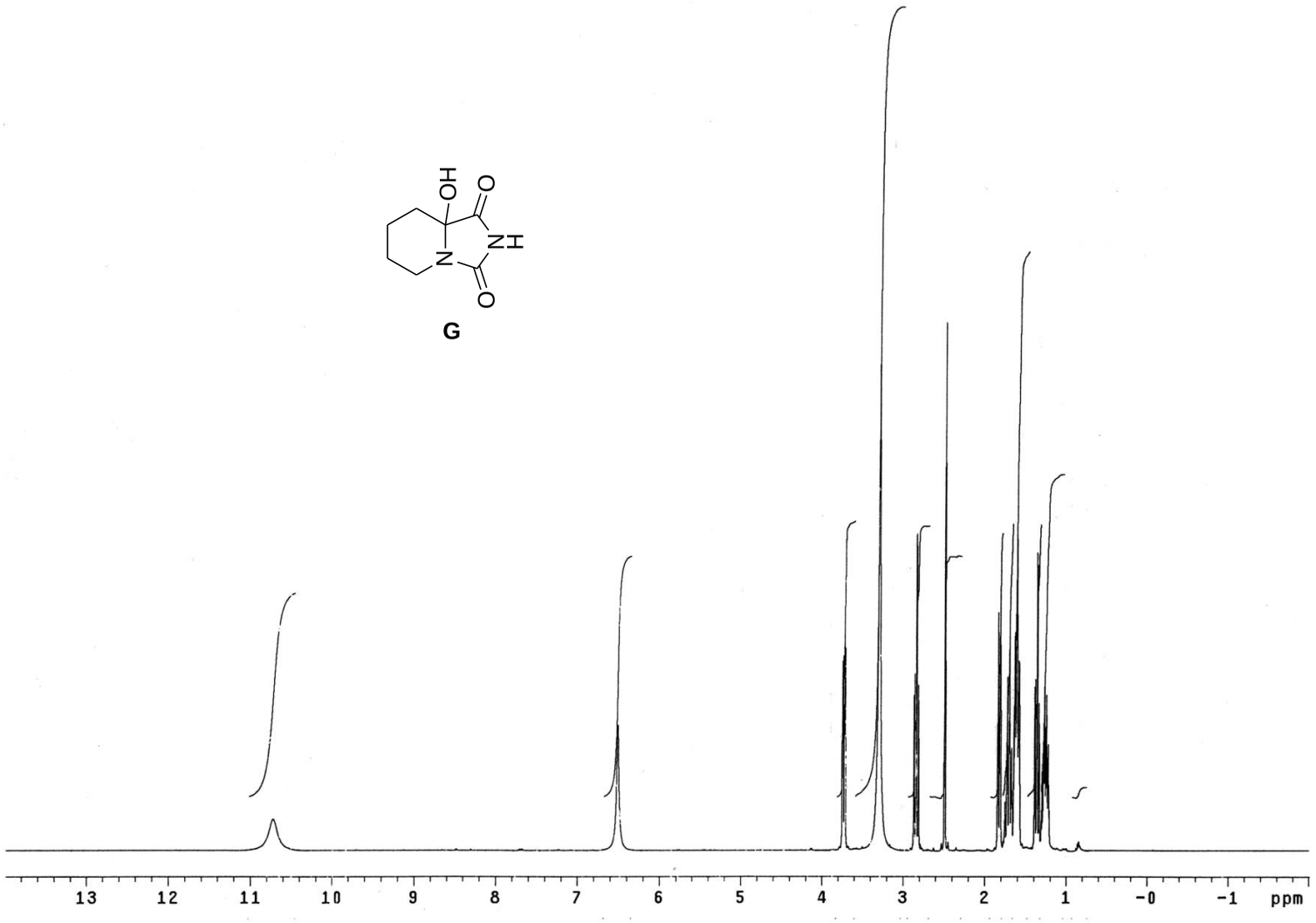


Figure 19. ^{1}H NMR spectrum of **G** (500 MHz, DMSO-d_6)

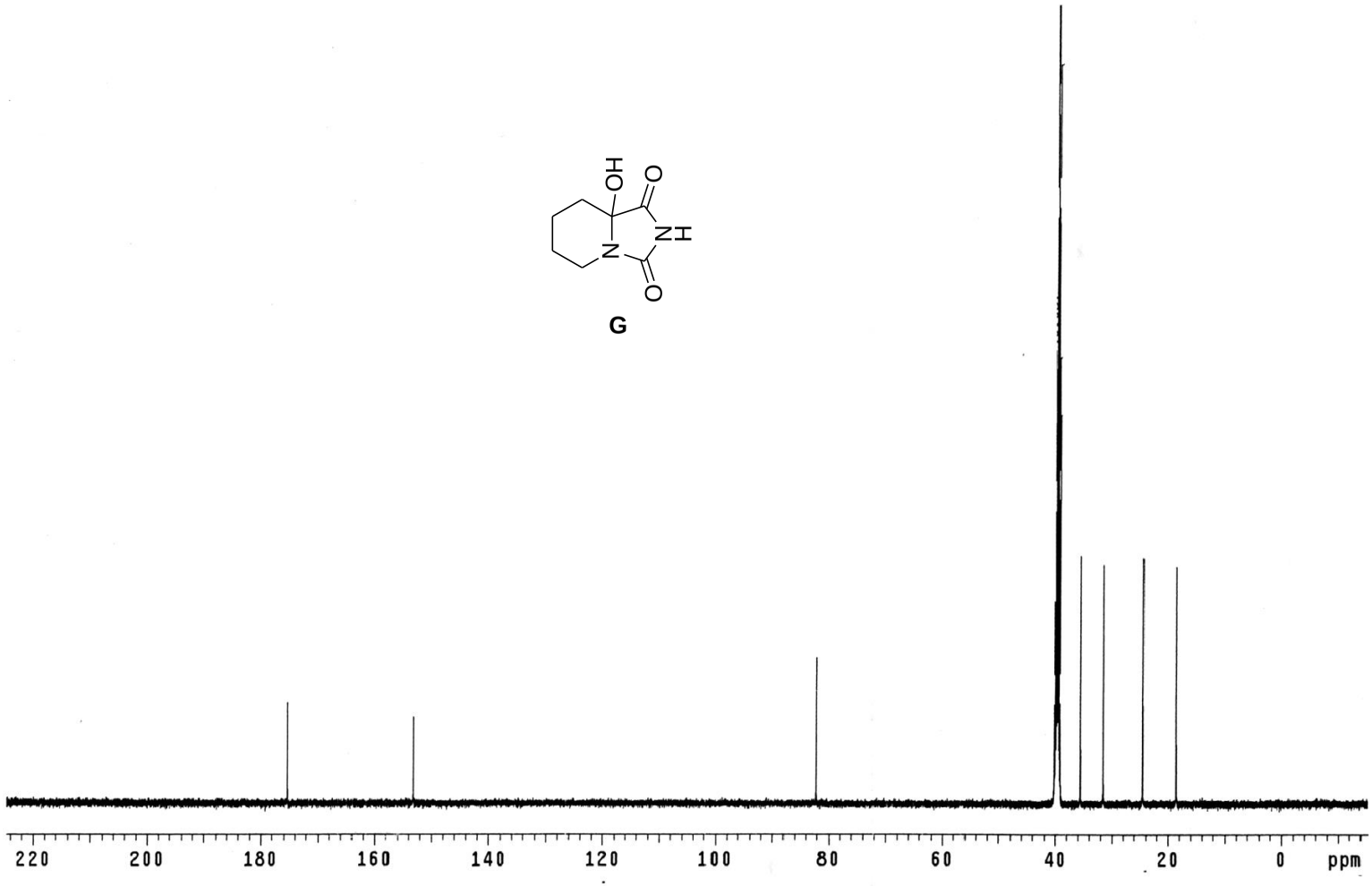
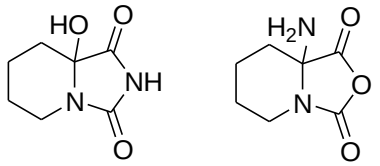


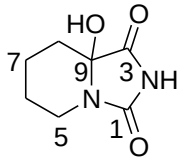
Figure 20. ^{13}C NMR spectrum of **G** (125 MHz, DMSO-d_6)

δ_H 1.83 (H-8a) and 1.36 (H-8b) to δ_C 175.3 (C-1),24.4 (C-6), 24.4 (C-7), and 82.1 (C-9); hence two possible structures **I** and **II** arose.



I **II**

Based on the presence of the hydroxyl group as indicated by the IR spectrum, and the characteristic chemical shifts of the hydatoin moiety at δ_C 175.3 (C-1) and 153.1 (C-3) (Divjak et al, 2009), the structure **I** was proposed for **G**. The compound was 2*H*,5*H*,7*H*,9*H*-9-hydroxy-imidazole[1,5-*a*]pyridine-1,3-dione, a transminated product of citrulline, previously reported by Cooper and Meister (1978).



G

Table 8. NMR data of G (500 MHz for ^{1}H and 125 MHz for ^{13}C; DMSO-d_6)

position	^{13}C (mult)	^{1}H (*J* in Hz)	HMBC correlation (C→H)
1	175.3 (C)		
2 NH	-	10.72, br s	
3	153.1 (C)		
5a	35.6 (CH_2)	3.73, br dd (13.1, 5.2)	C-3, C-6, C-7, C-9
b		2.83, ddd (13.1, 13.4, 3.4)	
6a	24.4 (CH_2)	1.62, overlap	C-7, C-8
b		1.26, br dddd (13.4, 13.4, 4.8, 4.8)	

Table 8. cont.

position	^{13}C (mult)	^{1}H (*J* in Hz)	HMBC correlation (C→H)
7a	24.4 (CH_2)	1.63, overlap	C-6, C-8
b		1.70, br dddd (13.4, 13.4, 4.8, 4.8)	
8a	31.5 (CH_2)	1.83, ddd (13.1, 4.8, 4.1)	C-1, C-6, C-7, C-9
b		1.36, ddd (13.1, 13.1, 4.1)	
9	82.1 (C)		
9-OH	-	6.50, br s	

3.3 Biological activities of the isolated compounds

All the compounds isolated from each project were subjected to the biological activity determinations (Table 9). For the diterpenes from the *Stylissa* cf. *massa* sponge, the antiplasmodial activity against *Plasmodium falciparum* and the antiproliferative activity against MCF-7 cell line were examined. As for the compounds from the soft coral *Eleutherobia* sp., the antiproliferative activity against KB and HeLa cell lines was tasted.

Table 9. Biological activities of isolated compounds

Compounds	Antiplasmodial activity (IC_{50}; µM)	Cytotoxicity (IC_{50}; µM)		
	P. falciparum	MCF-7	KB	HeLa
A	8.85	inactive	NT	NT
B	8.70	inactive	NT	NT
C	0.52	inactive	NT	NT
D	inactive	inactive	NT	NT
E	NT	NT	0.19	0.18
F	NT	NT	0.17	0.74
G	NT	NT	0.17	0.74

Table 9. cont.

Compounds	Antiplasmodial activity (IC_{50}; μM)	Cytotoxicity (IC_{50}; μM)		
	P. falciparum	**MCF-7**	**KB**	**HeLa**
Dihydroartemisinin	0.001	NT	NT	NT
Mefloquine	0.03	NT	NT	NT
Camptothecin	NT	0.002	0.002	0.07

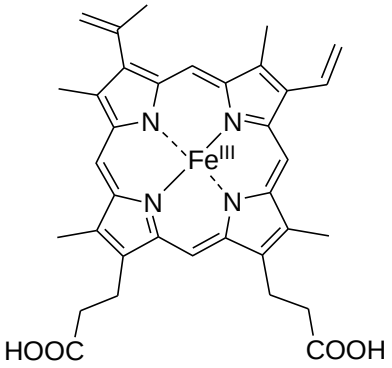
Note; NT = not tested, inactive = %inhibition less than 50% (32.9%, 17.2%, 29.7%, and 33.1% of **A**, **B**, **C**, and **D**, respectively).

Among the four diterpenes, compound **C**, was the most active, presumably due to the isonitrile functionality. On the other hand, compound **D**, lacking of the functional group that facilitate heme-complex formation, was inactive. The antiproliferative activity of all eight compounds reveal very weak activity of **A** - **D** and moderate activity of **E** - **G** (IC_{50}= 0.17 - 0.19 μM; KB cell, and 0.17 - 0.84 μM; HeLa cell)

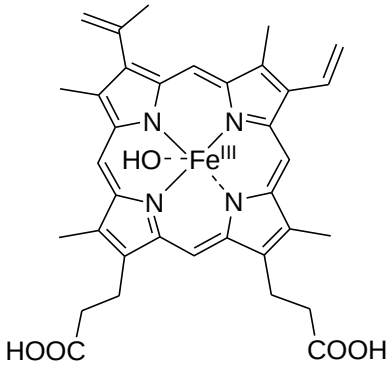
3.4 Spectroscopic characterization of heme-DIA complex

The antiplasmodial activity of compounds **A**, **B**, and **C** raised a question how the three compounds, as well as other amphilectene derivatives isolated from the same sponge specimens; i.e., 8-isocyanoamphilecta-11(20),15-diene, 7-isocyanoamphilecta-11(20),15-diene, and 8-isocyanoamphilecta-11(20),14-diene (Wattanapiromsakul et al, 2009), inhibit the growth of *Plasmodium* parasites. As described earlier in section 1.5, the complex formation between heme and the isonitrile and the other related functionalities have been proposed to play a major role in the antiplasmodial activity. In line with such observation, 8,15-diisocyanoamphilecta-11(20)-ene (DIA), which was the most active isonitrile amphilectene derivative (IC_{50}= 0.09 μM against *P. falciparum* K1; Wattapiromsakul et al, 2009) was selected as a model for this part of the investigation.

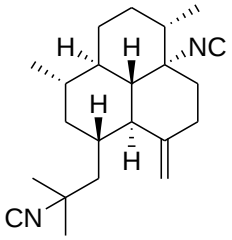
Possessing two isonitrile functionalities, DIA has been hypothesized that such functionality may participate in a heme-isonitrile complex, hence affecting the biocrystalization process that *Plasmodium* use to convert the toxic free-heme to the insoluble hemozoin (Kumar et al, 2007). The characterization of heme-DIA complex is explored here. A series of spectrometric/ spectrophotometric experiments, namely UV-visible absorption, emission, CD, ^{1}H NMR and mass spectrometry, were conducted. In addition, the complex formation between hemoglobin (Hb) and DIA was examined.



heme



hematin



8,15-diisocyanoamphilect-11(20)-ene (DIA)

3.4.1 Complex preparation

The complex between heme and DIA was prepared in 50%aq DMSO in various concentrations. The stock solutions of heme and DIA were freshly prepared using the stock solutions of hematin and DIA in DMSO. The using of hematin in this investigation allowed the

preparation of the complex solutions of heme and DIA to be in an aqueous medium. Heme complex was achieved in situ by losing of the hydroxyl group from hematin once DIA was added. The desired concentrations were acquired upon dilution with DMSO and water until the 50%aq DMSO condition was achieved and in order to mimic the aqueous condition of biological medium as closely as possible. Note that the contrasting hydrophobicity and hydrophilicity between DIA and heme challenged the selection of medium, and DMSO is opted to compromise the solubility of DIA vs heme. Also, it must be noted that the ratio of DMSO/H_2O (1:1) is the limitation in this work as the solution turned cloudy upon increasing aqueous ratio.

3.4.2 Spectroscopic measurement

UV spectra of the solution of heme and heme-DIA (heme/DIA; 1:0, 9:1, 4:1, 7:3, 3:2, 1:1, 2:3, 3:7, 1:4, 9:1) were obtained in a range of 300 - 800 nm (Figure 21). Heme solution show three maxima at 402 nm, 493 nm, and 625 nm (dark blue line), whereas that of DIA does not showed any transition in the visible range (400 - 800 nm). The absorbance at 402 nm of heme:DIA mixture decreased in accordance to the increasing ratios of DIA. On the other hand, the absorbances at 436 nm, 536 nm, and 567 nm were increased along with DIA ratios.

Emission spectra of heme-DIA complexes (1:0.5, 1:1, 1:2, 1:4, and 1:20; Figure 22) showed two emissions at 630 nm and 690 nm (excitation at 402 nm). Paralleled to the absorption spectra, emission intensity at 630 nm declined evidently when the heme:DIA mixture reached a molar ratio of 1:4 onward, and the emission at 690 nm otherwise emerged prominently at the 1:20 ratio.

The CD spectra of heme-DIA complex at a molar ratio 1:20 showed a positive Cotton effect at λ 430 nm, and a slight negative at λ 400 nm (Figure 23).

The paramagnetic effect of Fe(III) in heme prohibited the NMR phenomenon, in both of heme and heme-DIA complexes. However, titration of the solution of heme-imidazole complex (100 μM of heme, ratio 1:2 heme/imidazole) with DIA (1:2:0.5, 1:2:1, 1:2:2, 1:2:4

heme/imidazole/DIA) led to significant shifts of imidazole signals from 8.17 and 7.50 ppm to 8.11 and 7.40 ppm, respectively (Figure 24).

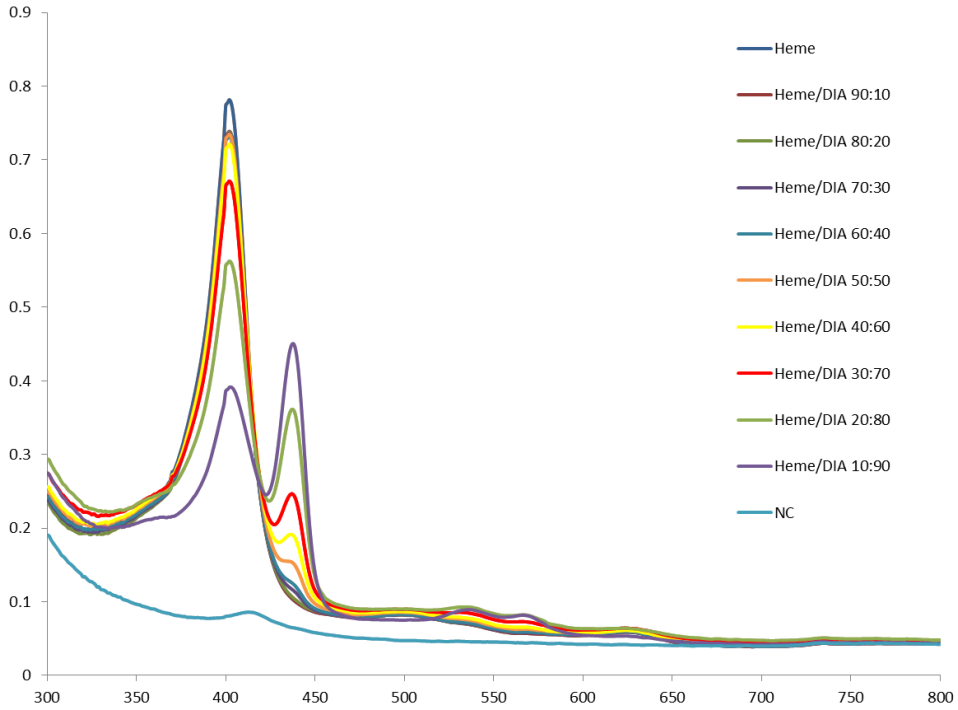


Figure 21. The UV-visible absorption spectra of heme (5 μM) and heme:DIA mixtures in 50%aq DMSO

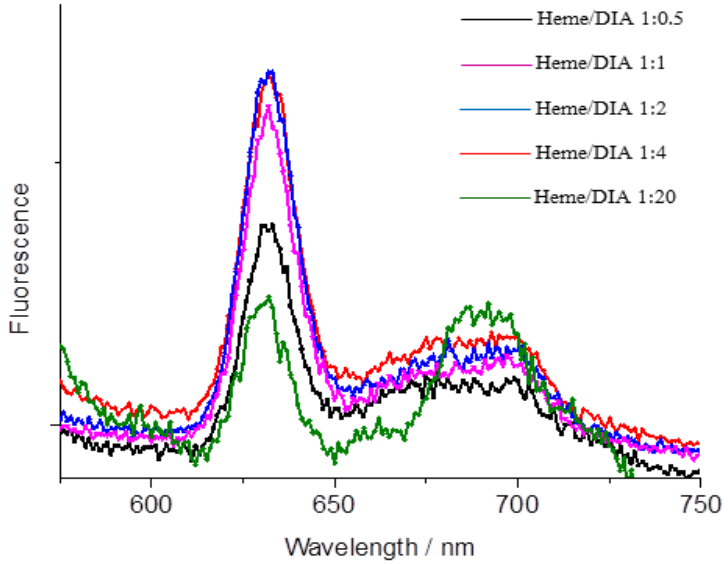


Figure 22. The emission spectra of heme (15 μM) and heme:DIA mixtures in 50%aq in DMSO (excited at 402 nm)

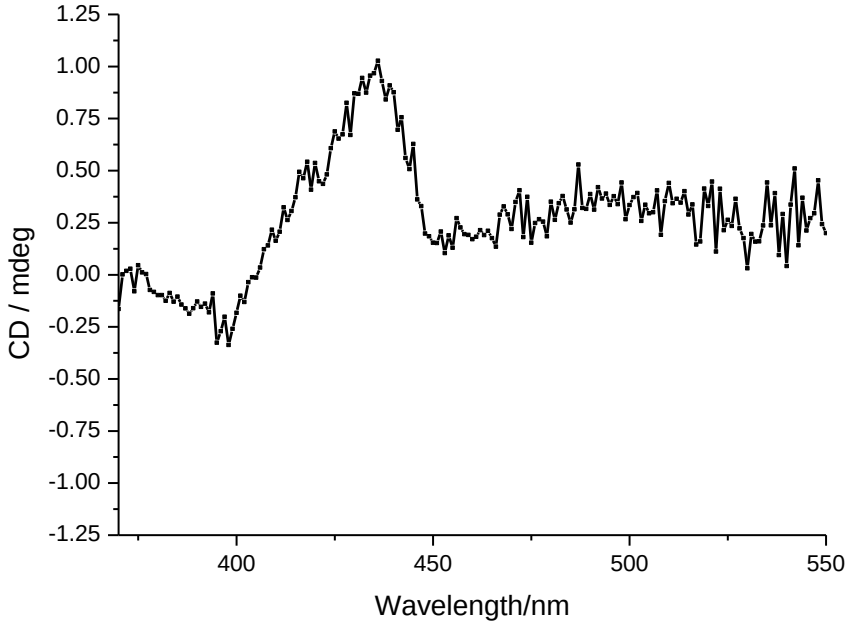


Figure 23. The CD spectrum of heme:DIA mixture 1:20 (100 μM of heme) in 50%aq DMSO

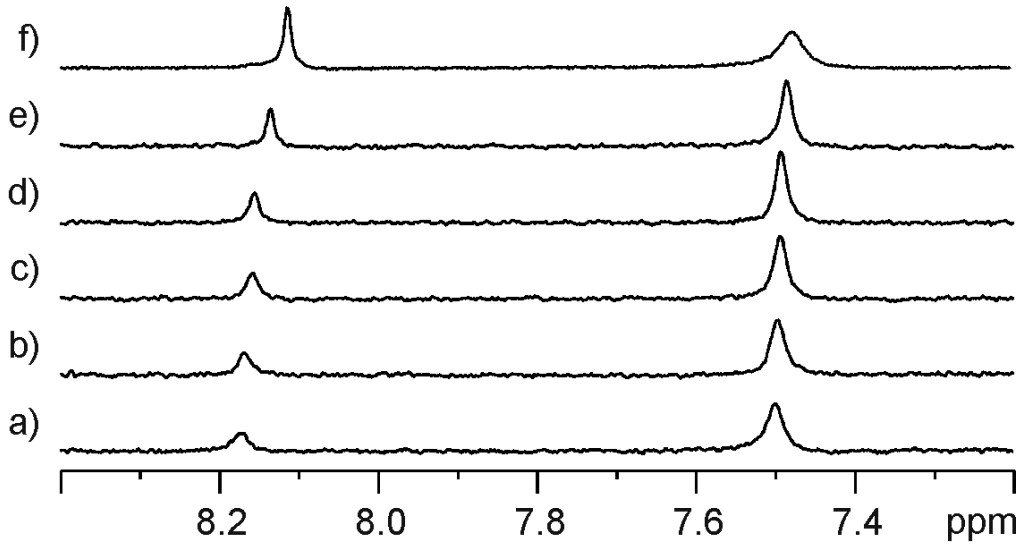


Figure 24. ^{1}H NMR Spectra of imidazole in heme/imidazole complex 1:2 (a) in 50% D_2O DMSO-d_6, (b) heme/imidazole/DIA 1:2:0.5, (c) heme/imidazole/DIA 1:2:1 (d), heme/imidazole/ DIA 1:2:2, (e) heme/imidazole/DIA 1:2:4, and (f) imidazole

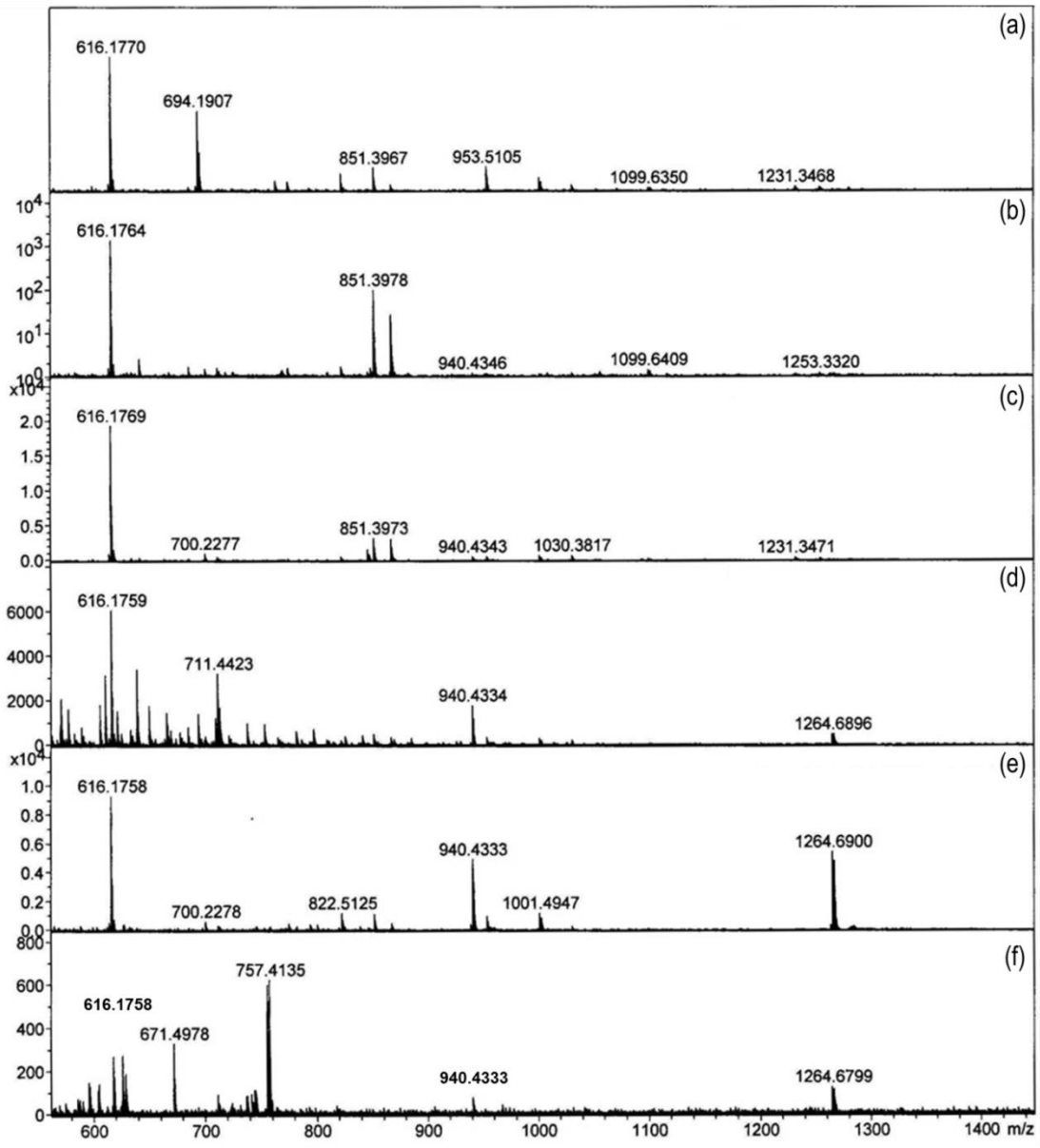


Figure 25. High resolution ESIMS spectra of heme (a); heme-DIA 1:0.5 (b); 1:1 (c), 1:2 (d); 1:4 (e) and 1:20 (f) in 50%D_2O DMSO-d_6 and dilution with CH_3CN/CH_3OH 1:1, containing 1% H_2O

The solutions of heme and heme/DIA complex at the molar ratios of 1:0.5, 1:1, 1:2, 1:4, and 1:20 were subjected to the ESI-mass spectrometric measurement using 1% H_2O in $MeCN/CH_3OH$ (1:1) as a dilution metrix. Heme yield significant peaks at *m/z* 616, 694, and 1231 whereas the mixtures showed major peaks at *m/z* 940 and 1264 (Figure 25).

3.4.3 Spectroscopic characterization of heme-DIA complex

The spectrophotometric- and spectrometric experiments as described in 3.4.2 substantially indicated the presence of heme-DIA complexes. The absorption spectra of heme in 50%aq DMSO showed Soret bands at 402 nm, 493 nm, and Q-band at 625 nm, resulting from the high-spin ferric complex, of which the fifth coordination incorporated weak ligand; i.e., either H_2O or DMSO (Santucci et al, 2001; Moreira et al, 2006).

Upon increasing the molar ratios of DIA, the intensity of the Soret band at 402 nm decreased, becoming evident at the ratio of 90:10 heme/DIA. The declining intensity in fact is a part of the bathochromic shift, and a new Soret band at 436 nm emerged. Notice that despite heavily excess of DIA (up to 1:20), no saturation was detected. The inability to reach plateau of the heme-DIA complex suggested a fast dynamic process, in which complex disintegration forbids the saturation of either 1:1 or 1:2 complexes.

In a parallel manner for the emission spectra, declining in emission band at 630 nm and increasing of 690 nm (excitation at 402 nm) upon the molar ratio between heme and DIA of 1:20 also supported the presence of the complex in both 1:1 and 1:2 ratios in the solutions. The CD spectrum of heme and an excess DIA at 1:20 ratio yielded the overlap of two CD spectra with the Cotton effect coherent to the UV absorption at 400 nm and 436 nm, hence adding a supporting evident to the heme-DIA complex formation. On the other hand, it could be presumed that the rate of reverse reaction for the complex is so fast that, unless highly excess of DIA was employed, the CD phenomenon is not detectable. Such necessity of high molar ratio of DIA could be true with the emerging emission band at 690 nm upon the reaching of 1:20 heme-DIA complex.

Along with a substantial proof for the presence of heme-DIA complex, the absorption spectra also suggested the possible orientation of DIA onto heme. A charge-transfer bands at 536 nm and 567 nm in the absorption spectra are responsible to the electronic transition of half-filled *d*-orbital of Fe(III) by the ligand. This indicated axial orientation of DIA to Fe(III) in the square planar heme (Goto et al, 2012; Bellemare et al, 2009).

It must be noted here that the measurement for the IR spectrum has been attempted. However, no transformations were observed when dry methods, i.e., either neat or KBr pellet, were applied, and with the hydroxy and sulfoxide stretching in the aqueous DMSO solution, the solution IR experiment was not fruitful. It could be speculated that, regardless of bond strength, a dry complex was not achieved, and the complex disintegrated upon dryness. However, the complex is stable to a certain extent, allowing the ionization to take place, and the ESI mass spectra can therefore be obtained. With the mass of both 1:1 and 1:2 complexes detectable (*m/z* 940 and 1264), this strongly indicated the existence of heme-DIA complexes in both ratios, and suggested that the complexes were stable at least in a solution and in a gas phase.

The determination of the orientation of DIA onto Fe(III), and changes in the conformation of either heme or DIA were trialed, particularly by means of NMR experiments. Unfortunately, the paramagnetic effects of Fe(III) forbade the NMR detection. The replacement of imidazole on heme-imidazole complex upon addition of DIA however supported the complex formation, and may open-up a possibility to determine the binding constant of heme-DIA complex.

3.4.4 Spectroscopic characterization of hemoglobin (Hb)-DIA complex

The ability of DIA to form complex with heme raised a question whether DIA may form complex with hemoglobin (Hb) in a similar manner to heme. In other words, it could be of interest to determine whether DIA might be used in human without affecting intrinsic hemoglobin. Similar experiments on the absorption, emission, and CD spectrophotometries were performed with Hb:DIA mixtures. However, no strong evidences in changing of the heme spectra were observed. The absorption spectra of Hb:DIA showed a Soret band at 406 nm (Figure 26). The slight humps at 436 nm were detected when the mixtures at the 1:4, 1:10, and 1:20 ratios of Hb/DIA were applied. However, with little changes in intensity, this may merely support the bleaching of heme upon adding the excess of DIA.

Despite changes in the intensity of emission and Cotton effects in the emission and CD spectra (Figures 27 and 28), at the moment, the changes in the intensity, particularly for that in CD spectra, were unable to be accounted for. Without shifting in the relevant wavelength, it is reasonable to dismiss the possibility of the Hb-DIA complex formation, but rather bleaching of heme out of Hb molecule.

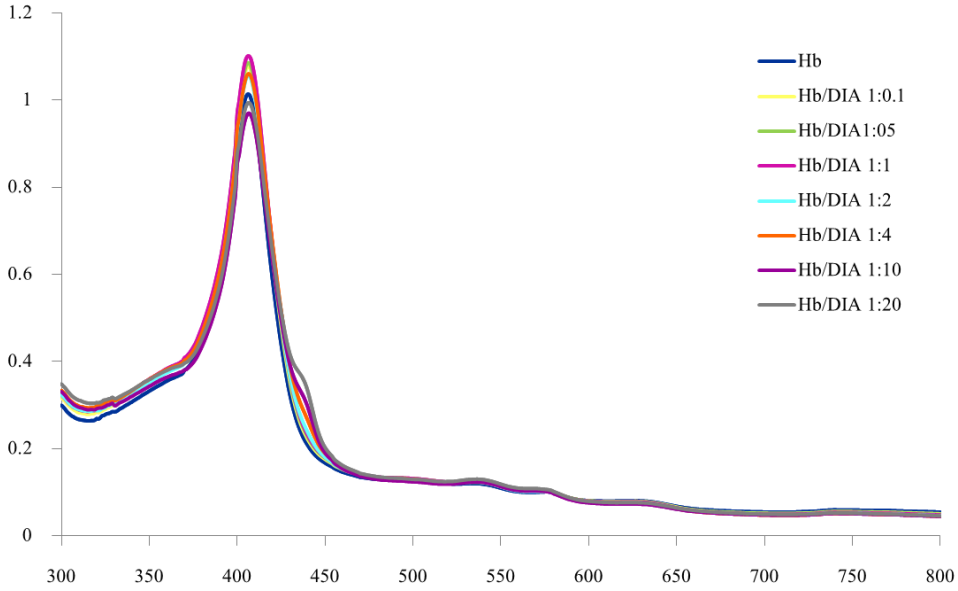


Figure 26. The UV-visible absorption spectra of Hb (2.5 μM) and of Hb:DIA mixtures in 50%aq DMSO

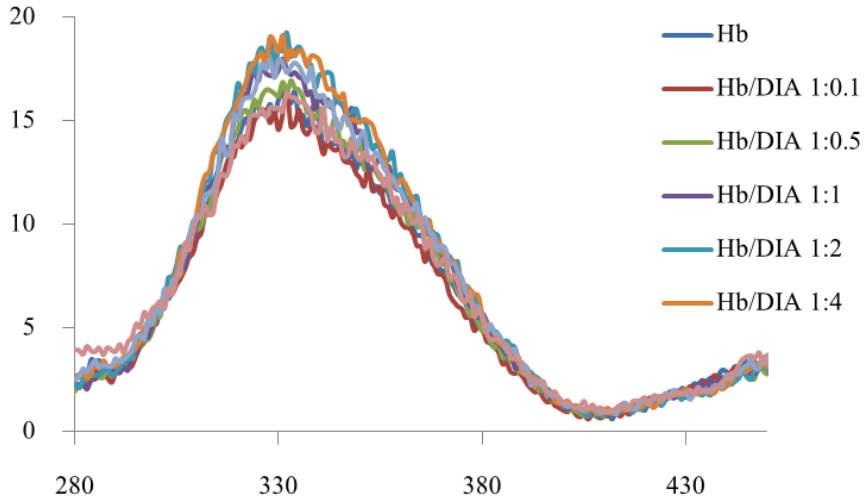


Figure 27. Emission spectra of Hb (10 μM) and Hb:DIA mixtures in 10% DMSO in water (excitation at 280 nm)

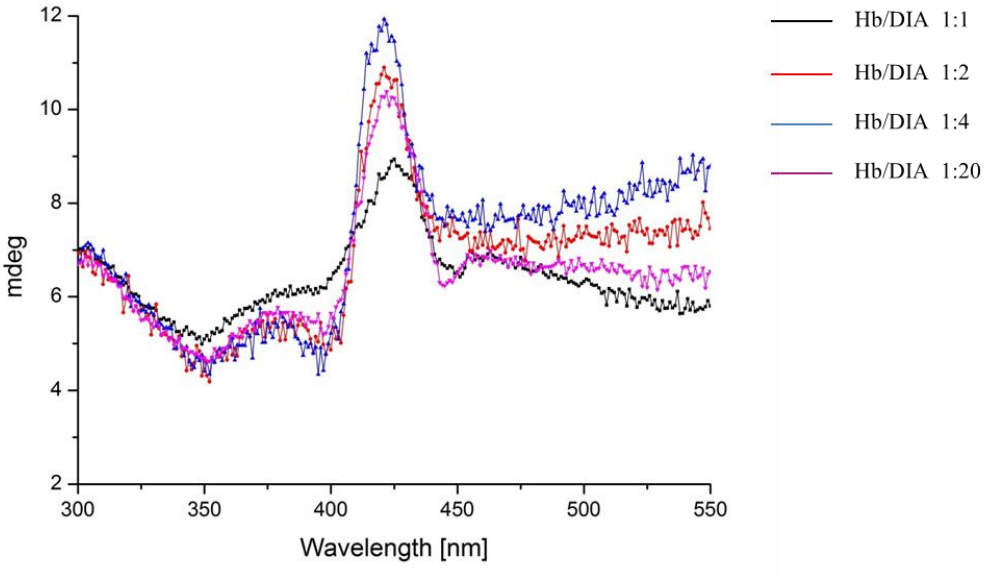


Figure 28. CD Spectra of Hb (100μM) and Hb:DIA mixtures in 10% DMSO in water

CHAPTER 4

CONCLUSION

In this dissertation, two independent researches have been conducted; the chemical investigation of the antiplasmodial compounds from the sponge *Stylissa* cf. *massa*, and the investigation toward the antiproliferative compounds from the soft coral *Eleutherobia* sp. The potential mechanism of the isonitrile diterpene as an antimalarial agent has also been studied.

Four diterpenes (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-8-isocyanato-15-formamido-amphilect-11(20)-ene (**A**), (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-8-isothiocyanato-15-formamido-amphilect-11(20)-ene (**B**), (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-8-isocyano-15-formamidoamphi-lect-11(20)-ene (**C**), and (1*S**,3*S**,4*R**,7*S**,8*S**,12*S**,13*S**)-7-formamidoamphilecta-11(20),15-diene (**D**), were isolated from *S. massa* sponge. Among those, compounds **A** and **B** are the new amphilectane-type diterpenes, on which two functional groups, formamide and either isocyanate or isothiocyanate are residing. Compound **C** showed the most potent antiplasmodial activity (IC_{50} = 0.52 μM) and compound **D** was inactive through this antiplasmodial assay.

The investigation on the soft coral *Eleutherobia* sp. led to the isolation of three compounds, xeniolide A (**E**), thymine (**F**), and 2*H*,5*H*,7*H*,9*H*-9-hydroxy-imidazole[1,5-*a*] pyridine-1,3-dione (**G**). All the isolated compounds showed the moderated cytotoxicity against KB and HeLa cell lines (IC_{50} = 0.19, 0.17, and 0.17 μM for KB cell, and 0.18, 0.74, and 0.74 μM for HeLa cell, respectively).

The mechanisms of 8,15-diisocyanoamphilecta-11(20)-ene (DIA) as an anti-plasmodial agent were evaluated. DIA forms both 1:1 and 2:1 complexes with heme, and the evidences can be spectroscopically observed. This is a leading and substantial evidence for the isonitrile diterpenes as a hemozoin biocrystallization preventer, allowing toxic free-heme to be available thus causing cellular damages to the *Plasmodium* parasites. The findings open up an opportunity for the development of DIA as drugs lead for new antimalarial agents.

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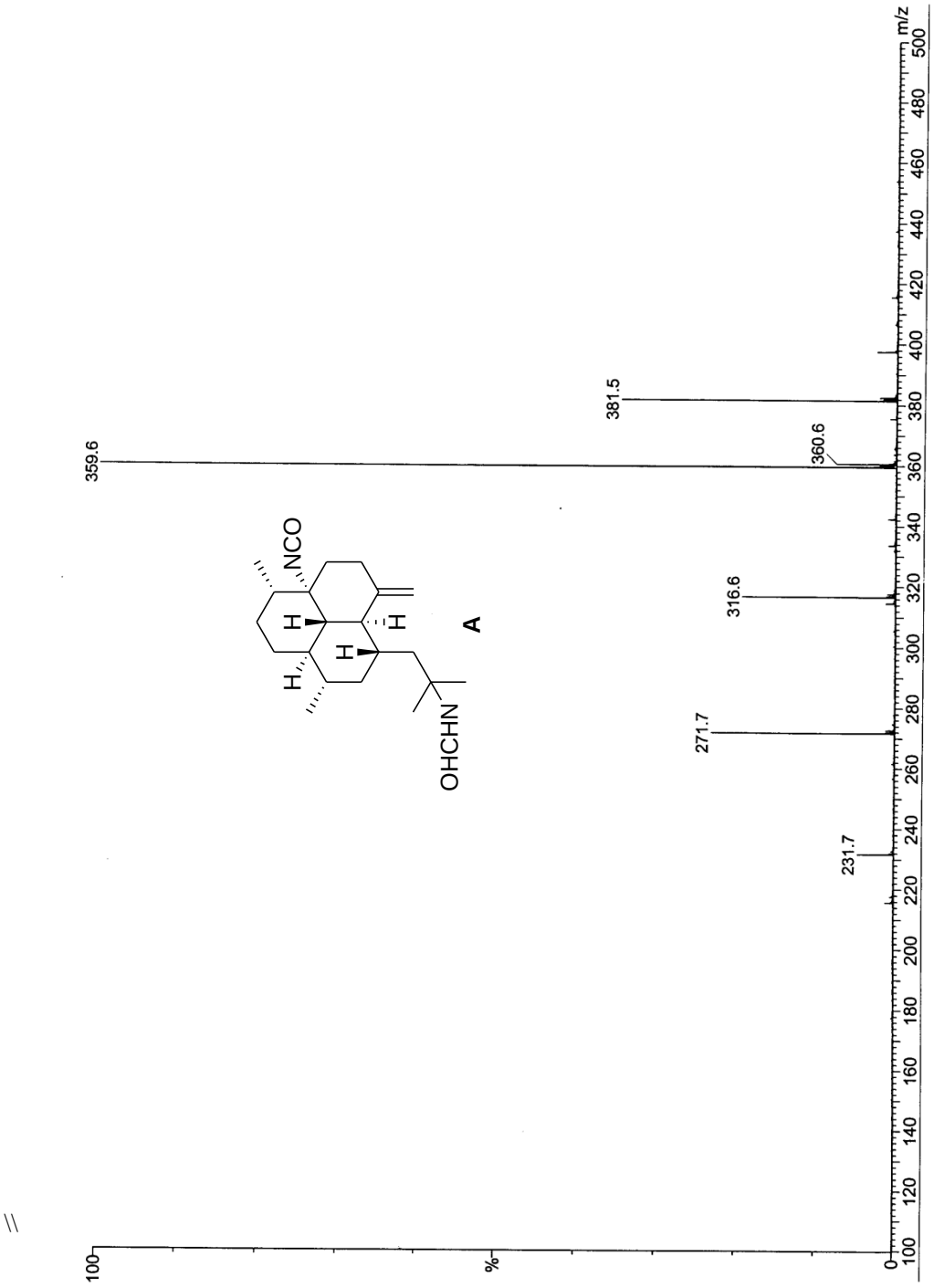
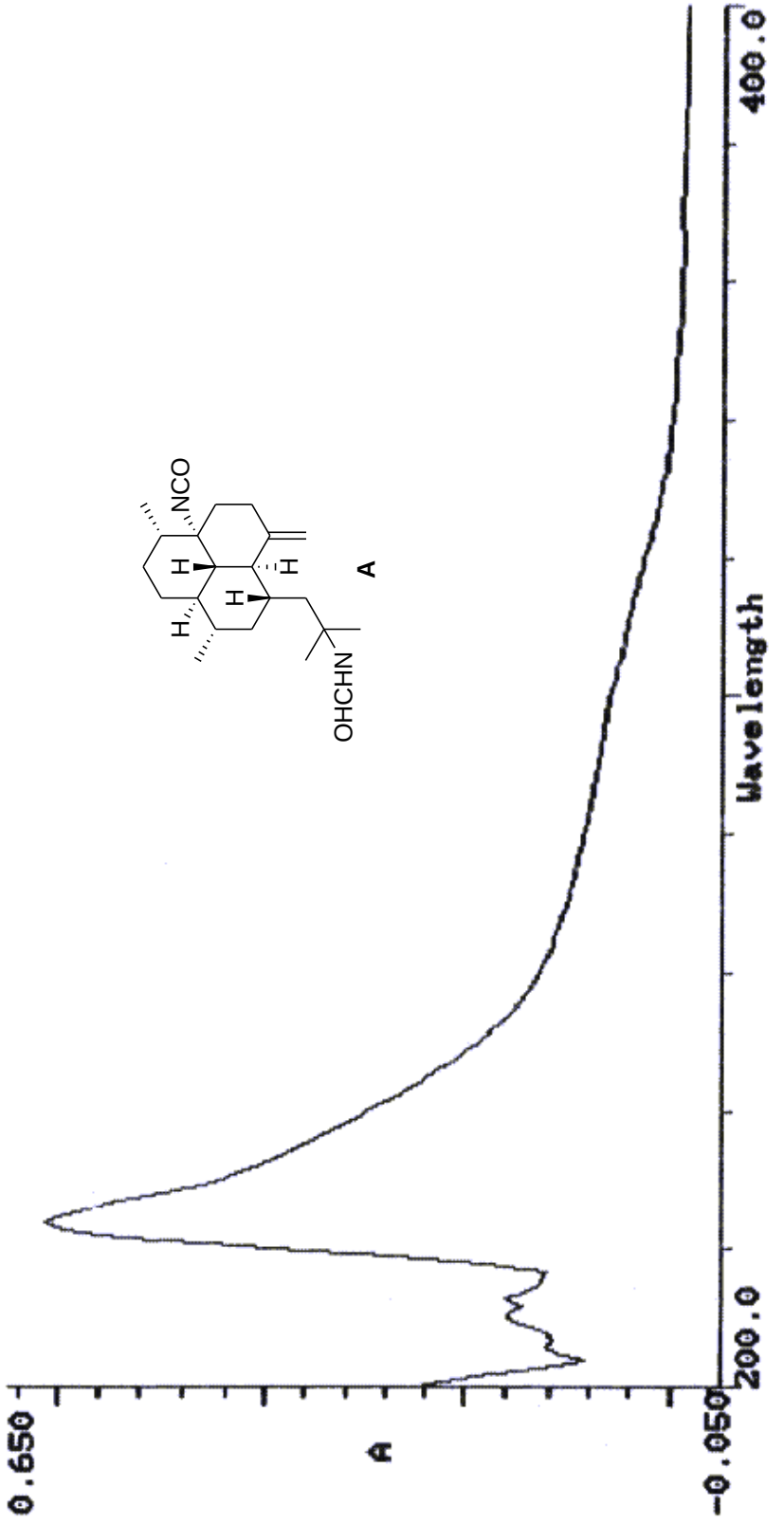
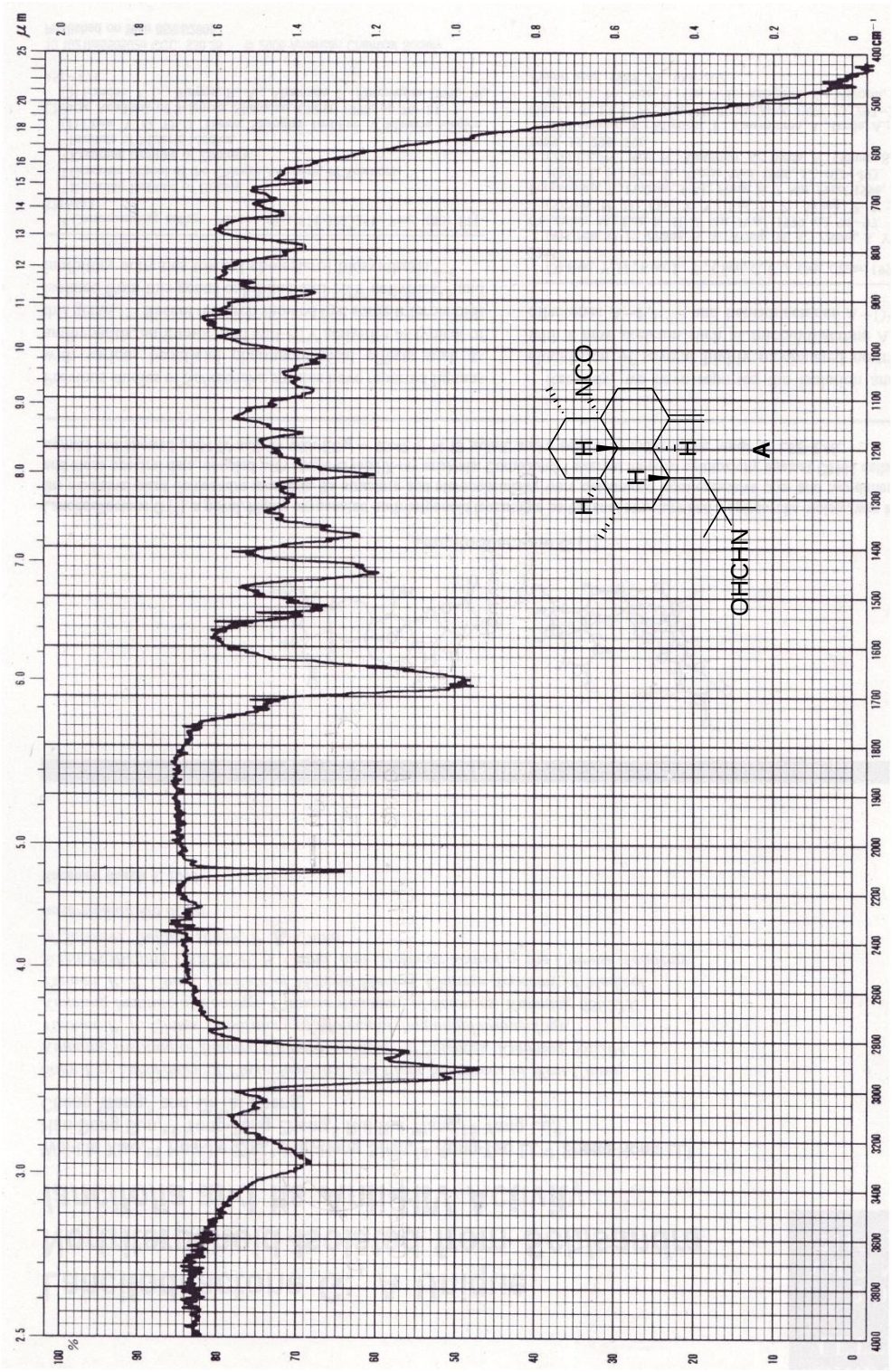
APPENDIX



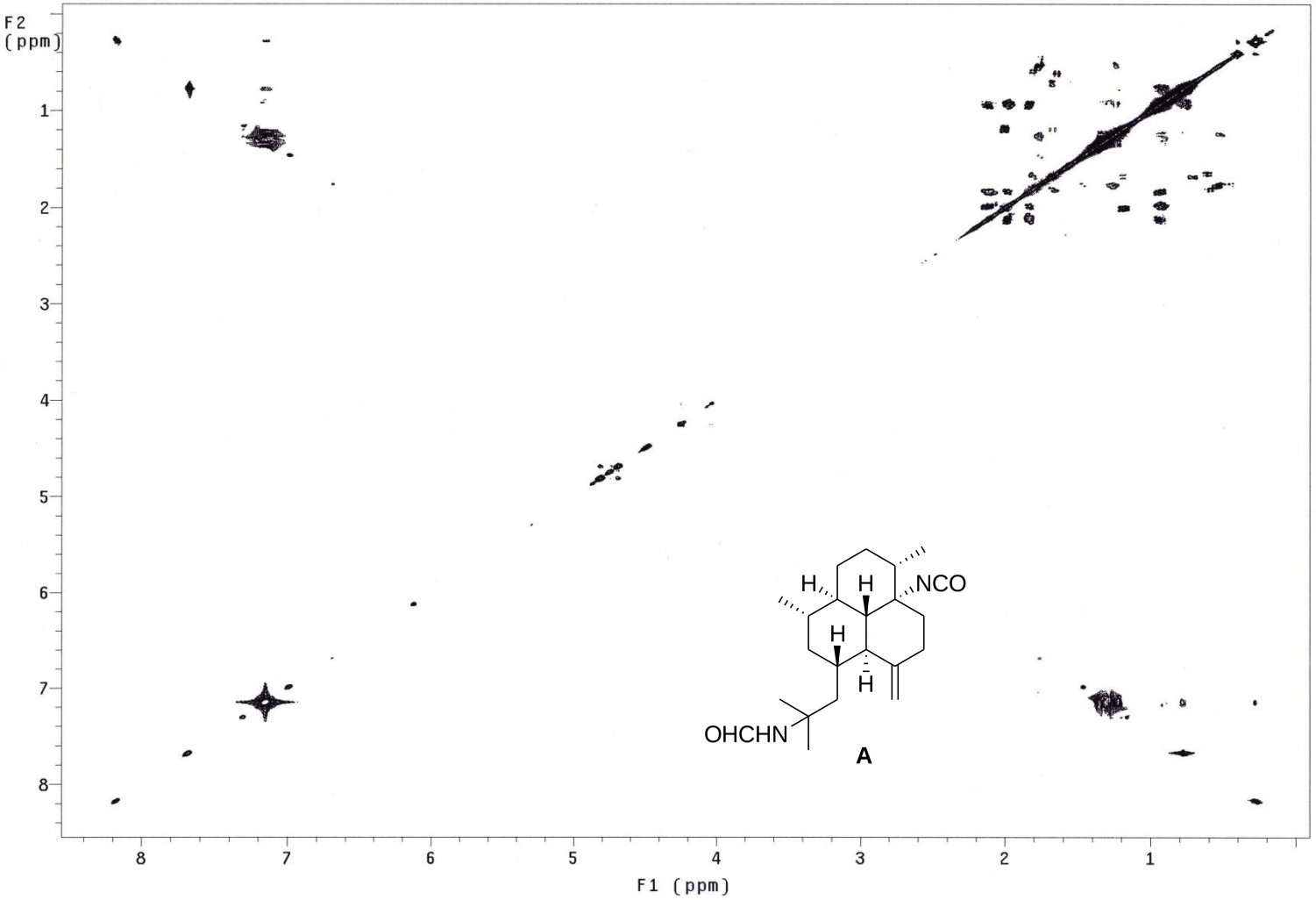
ESIMS spectrum of **A**



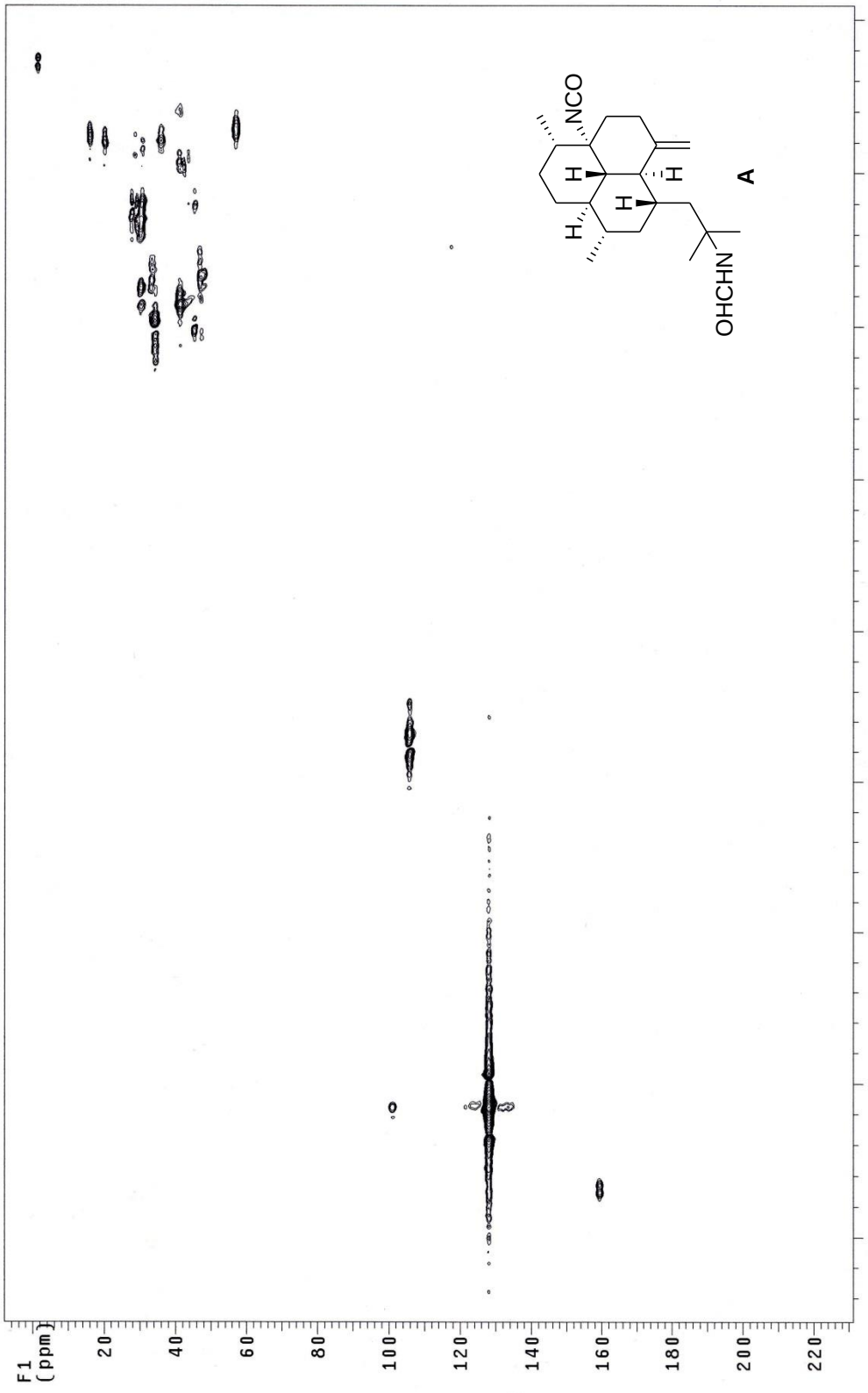
UV spectrum of **A** (CH_2Cl_2)



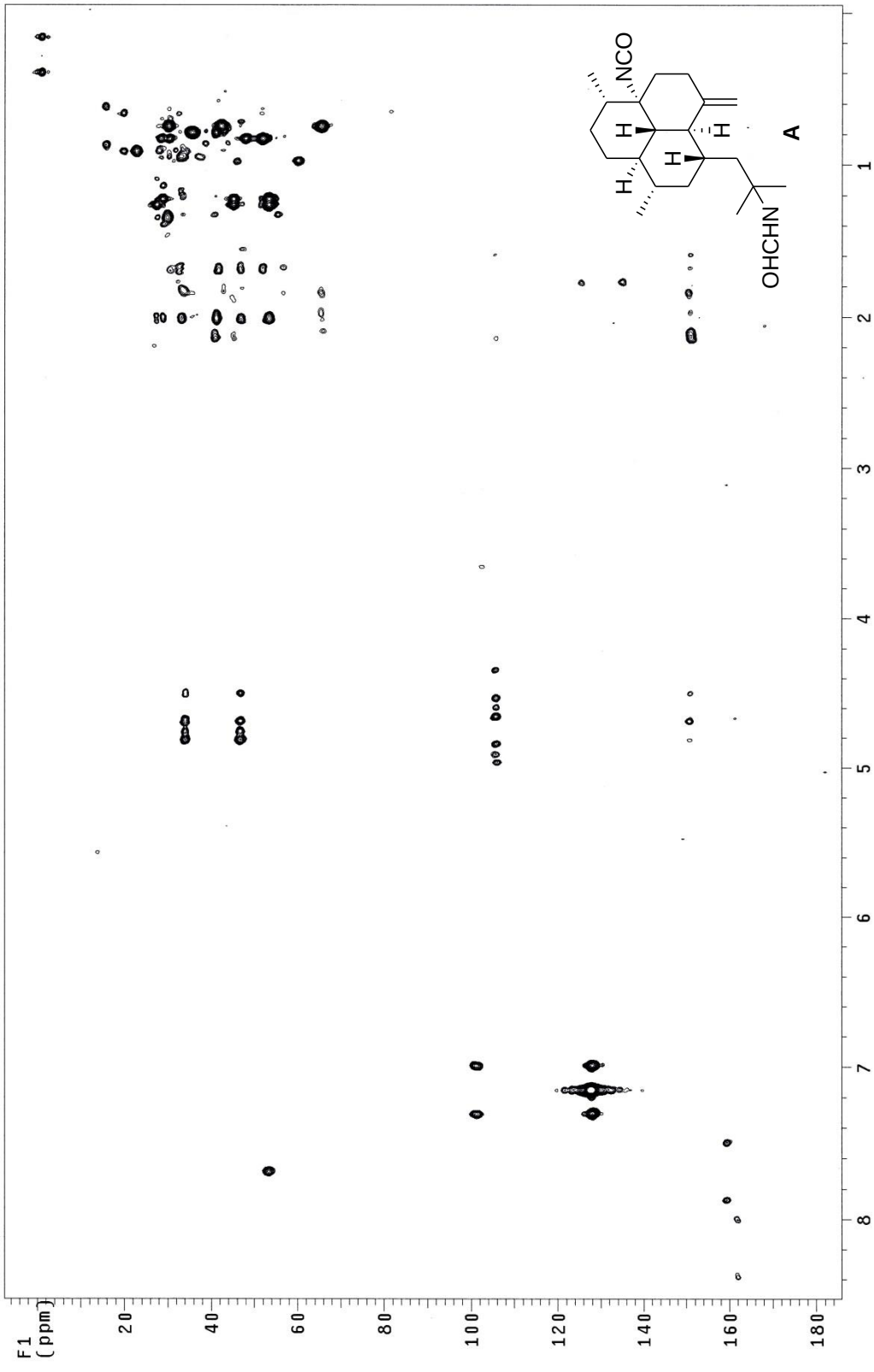
IR spectrum of **A** (thin film)



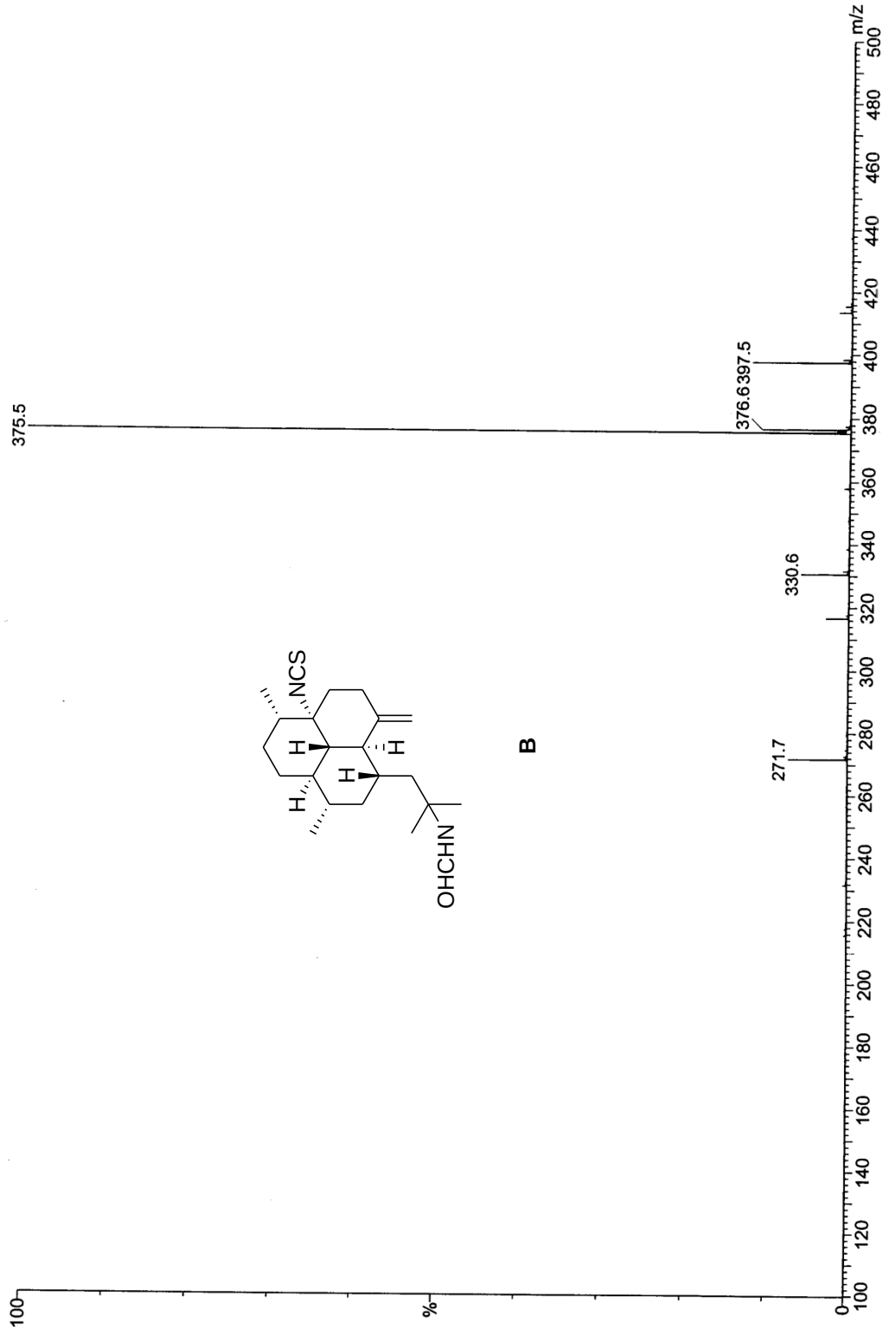
^{1}H, ^{1}H-COSY spectrum of **A** (500 MHz, C_6D_6)



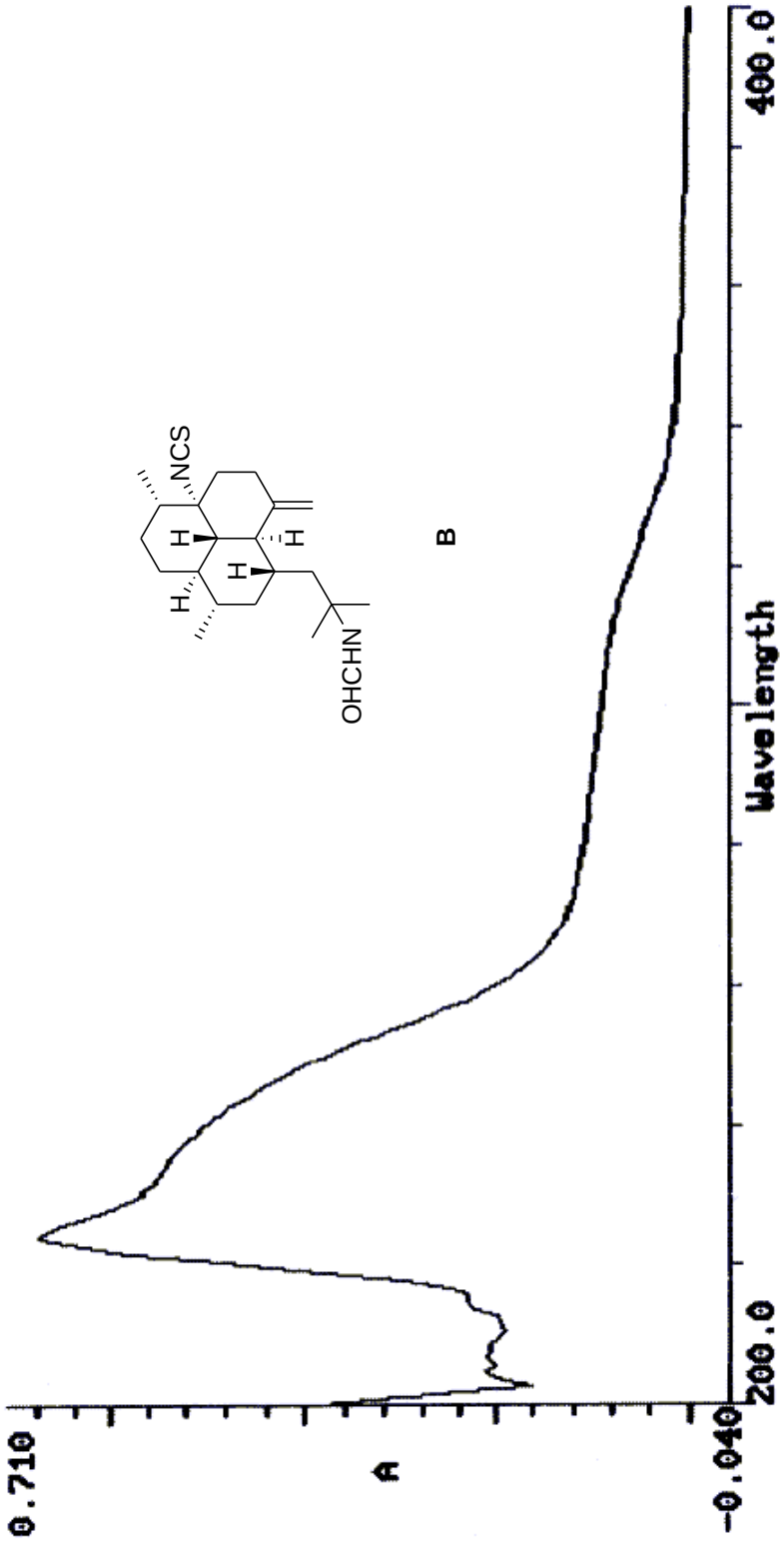
HMQC spectrum of **A** (500 MHz, C_6D_6)



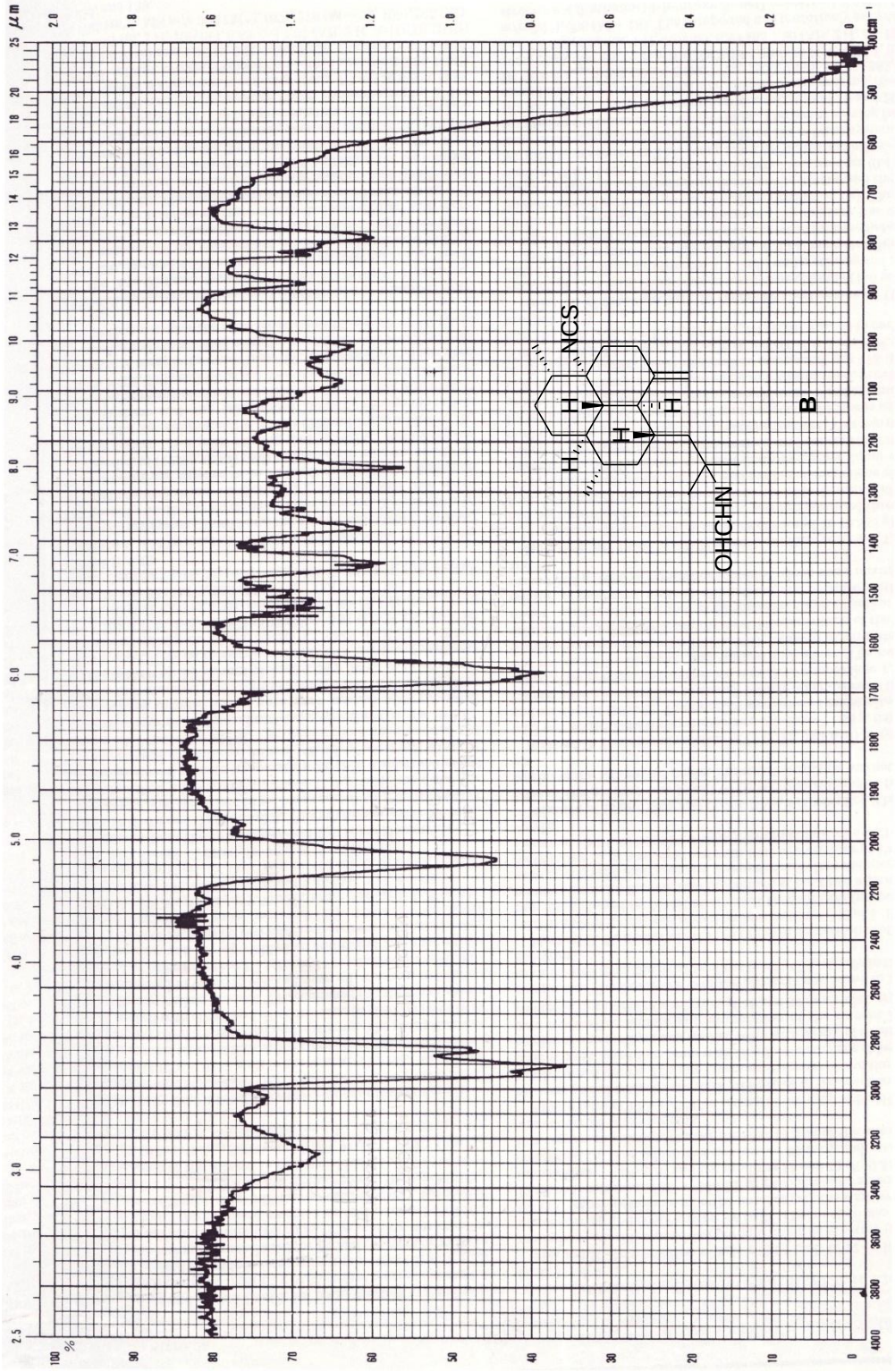
HMBC spectrum of **A** (500 MHz, C_6D_6)



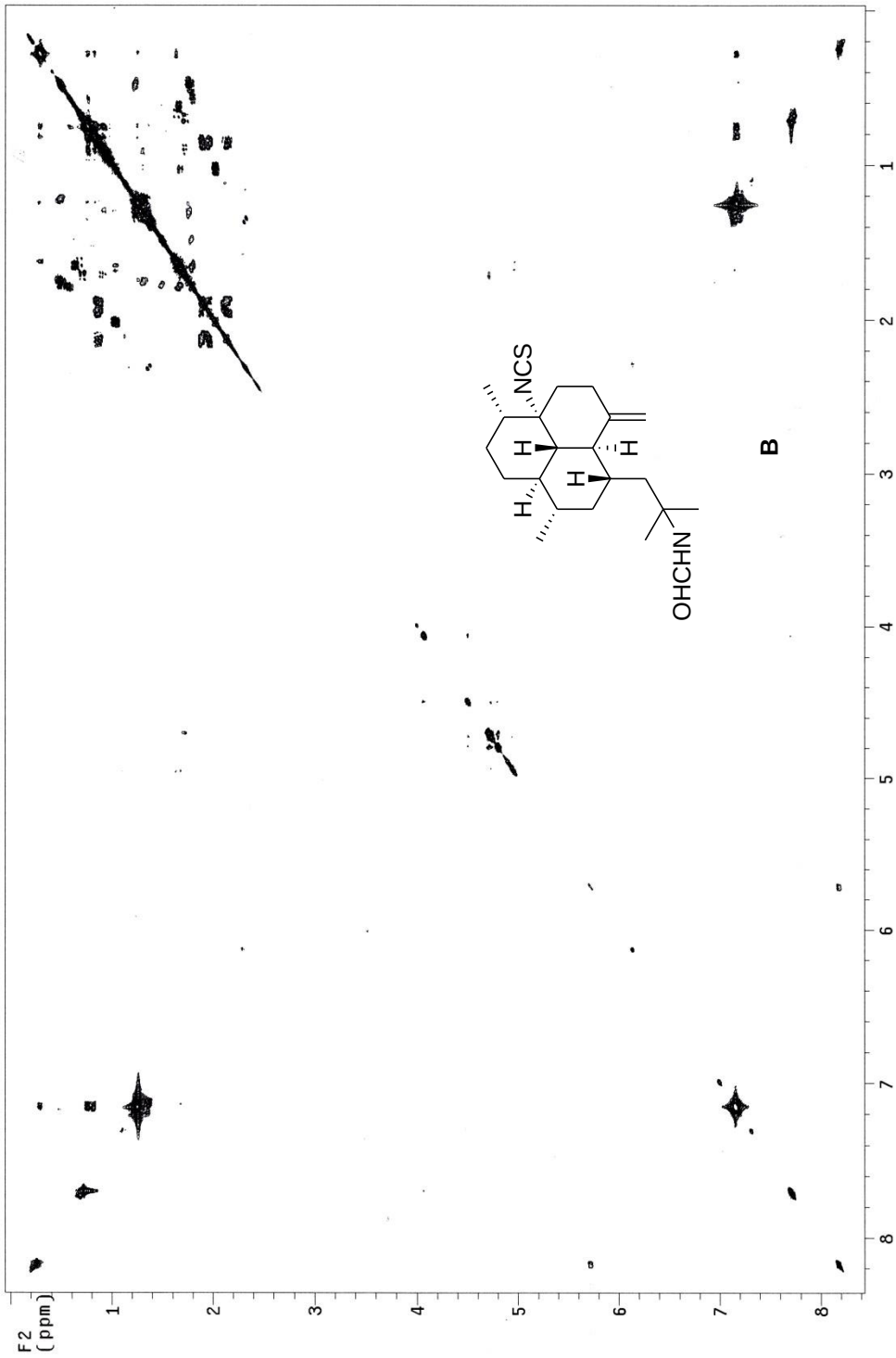
ESIMS spectrum of **B**



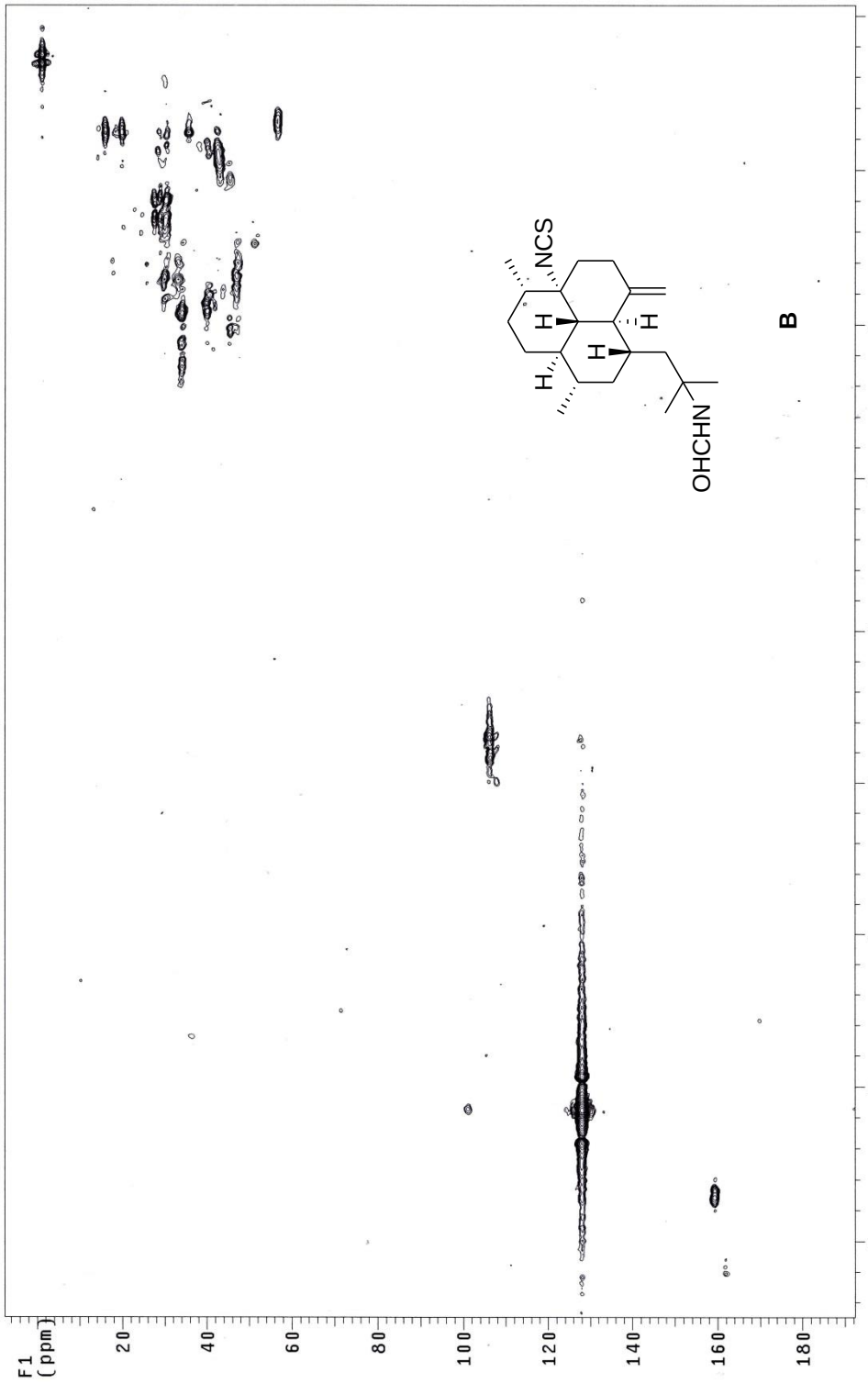
UV spectrum of **B** (CH_2Cl_2)



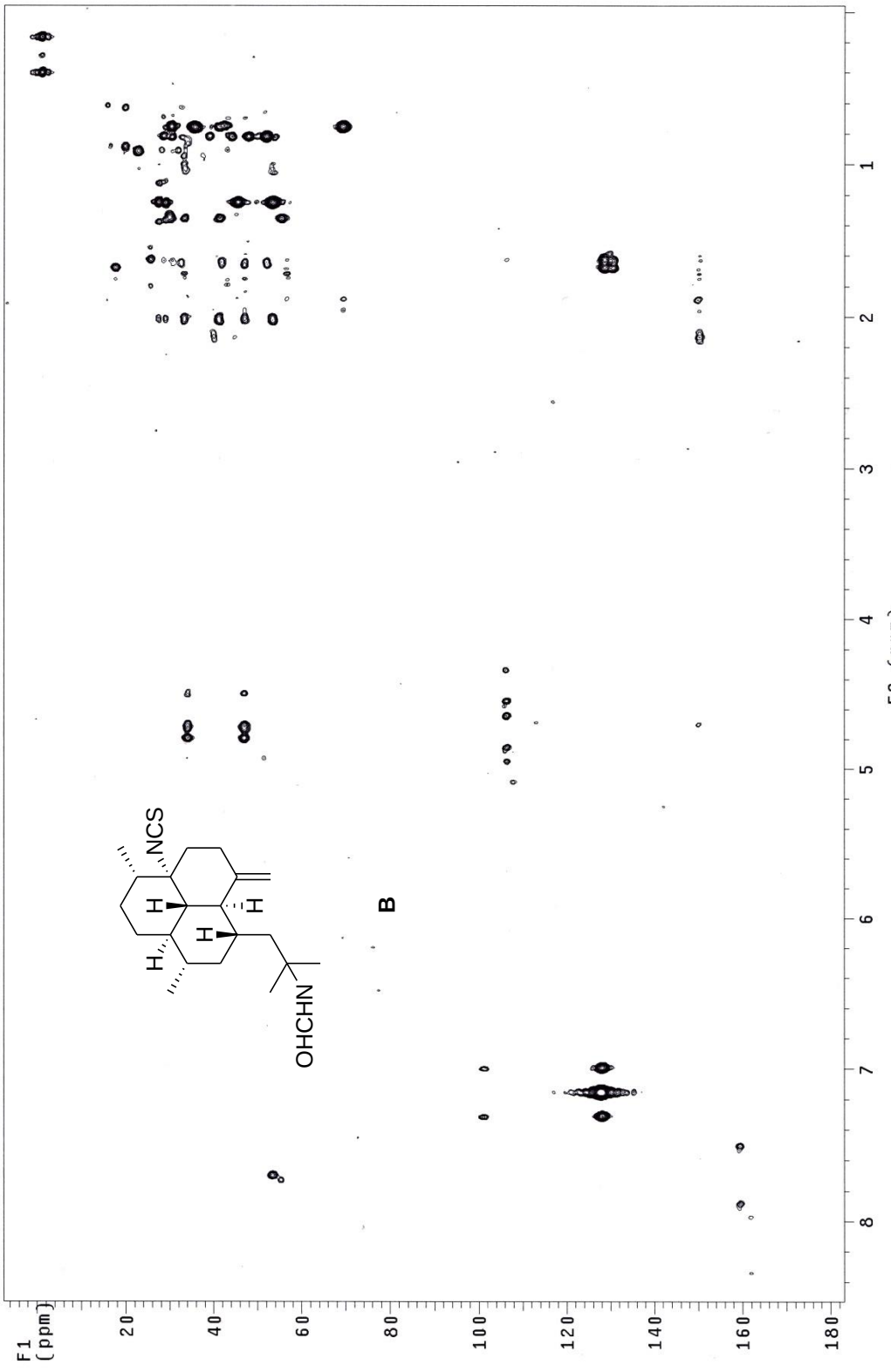
IR spectrum of **B** (thin film)



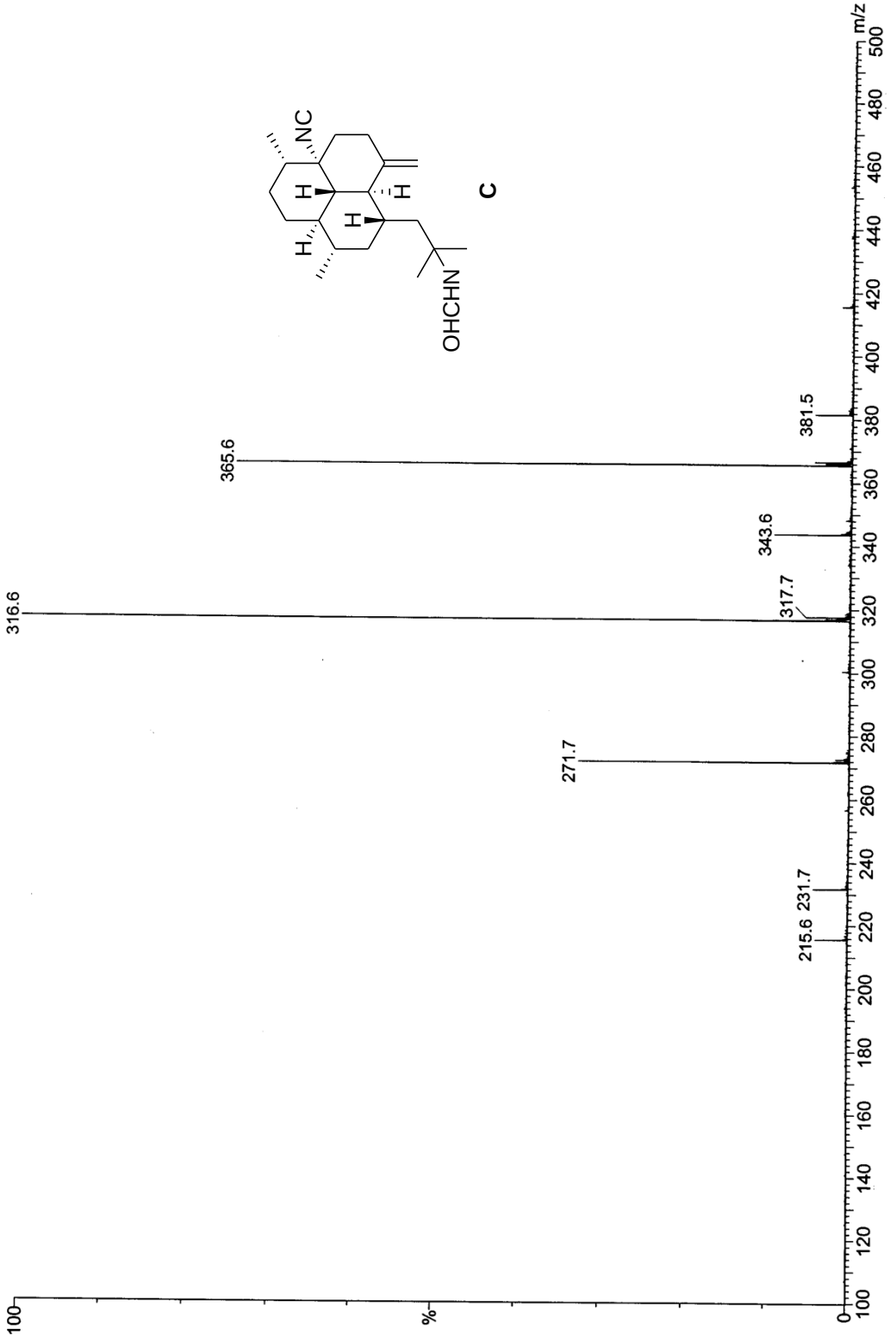
^{1}H, ^{1}H-COSY spectrum of **B** (500 MHz, C_6D_6)



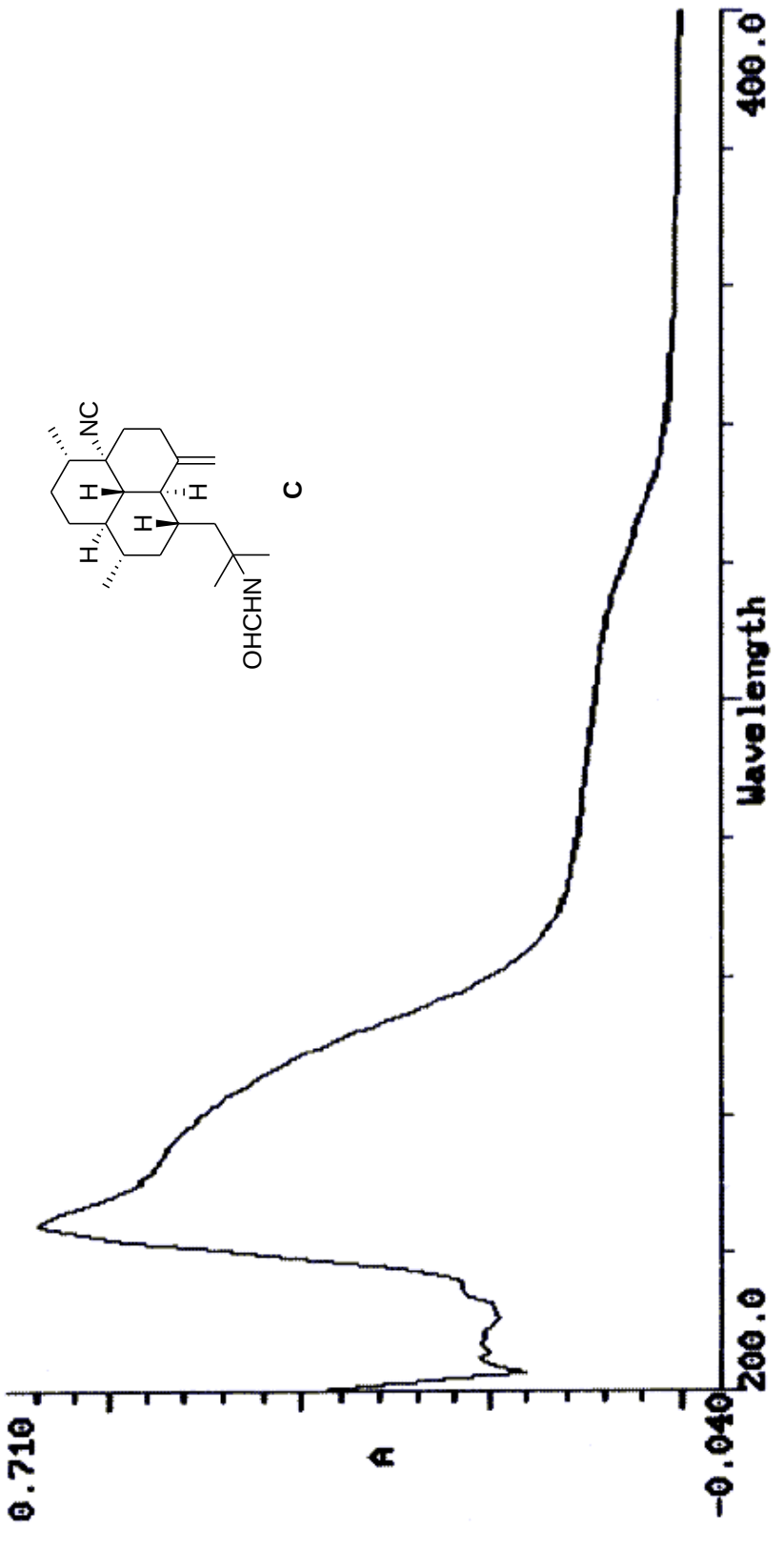
HMQC spectrum of **B** (500 MHz, C_6D_6)



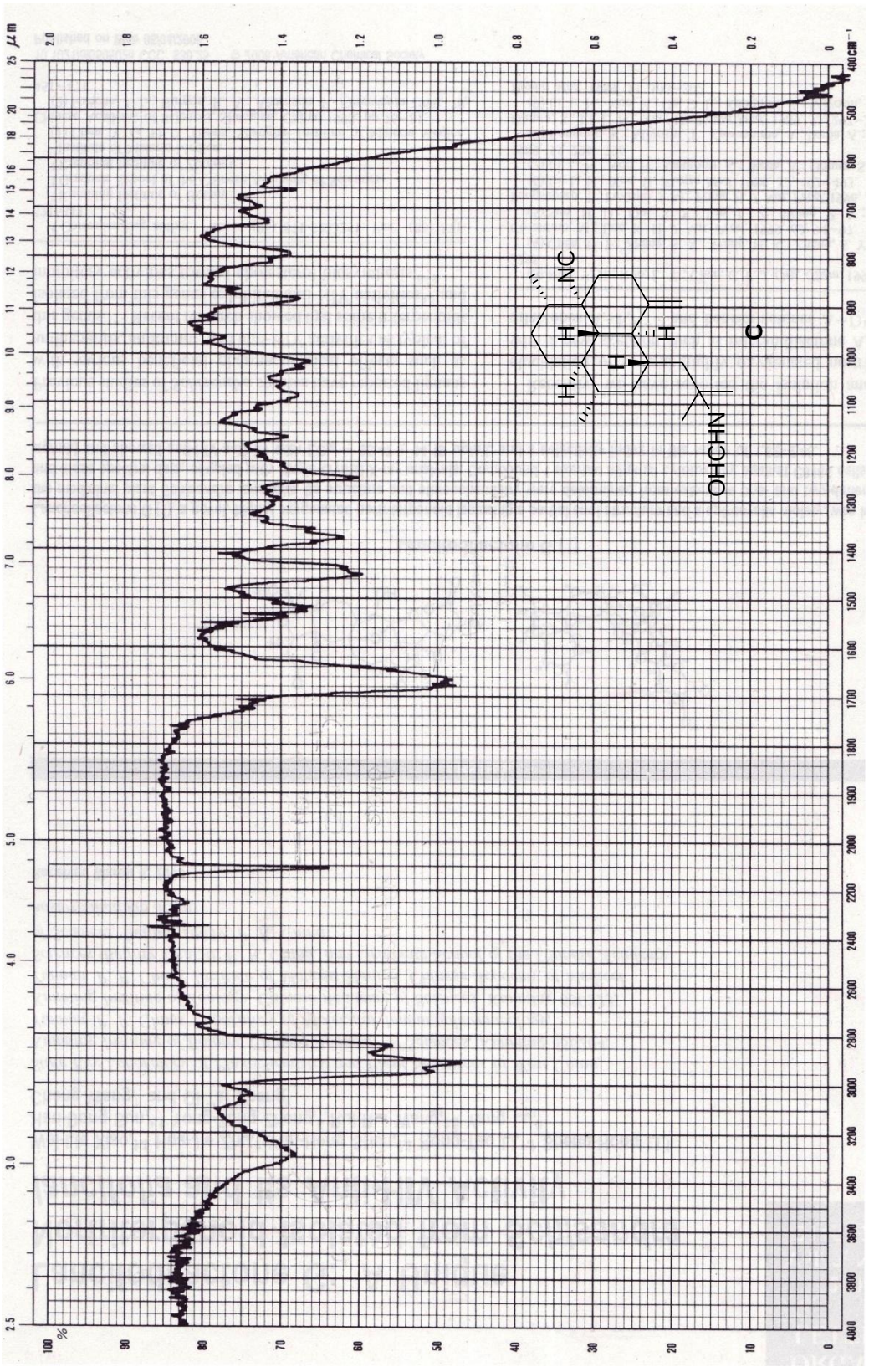
HMBC spectrum of **B** (500 MHz, C_6D_6)



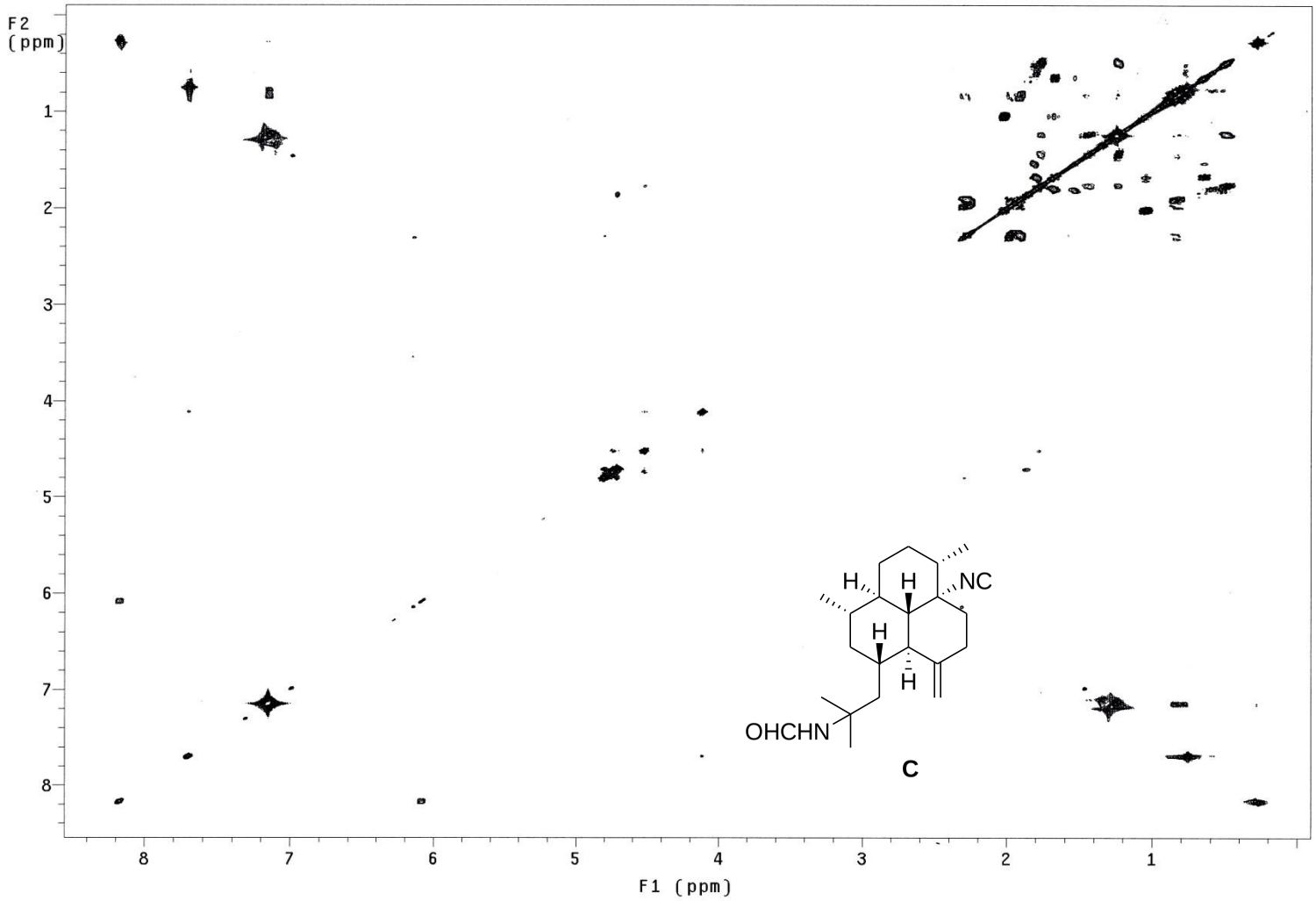
ESIMS spectrum of **C**



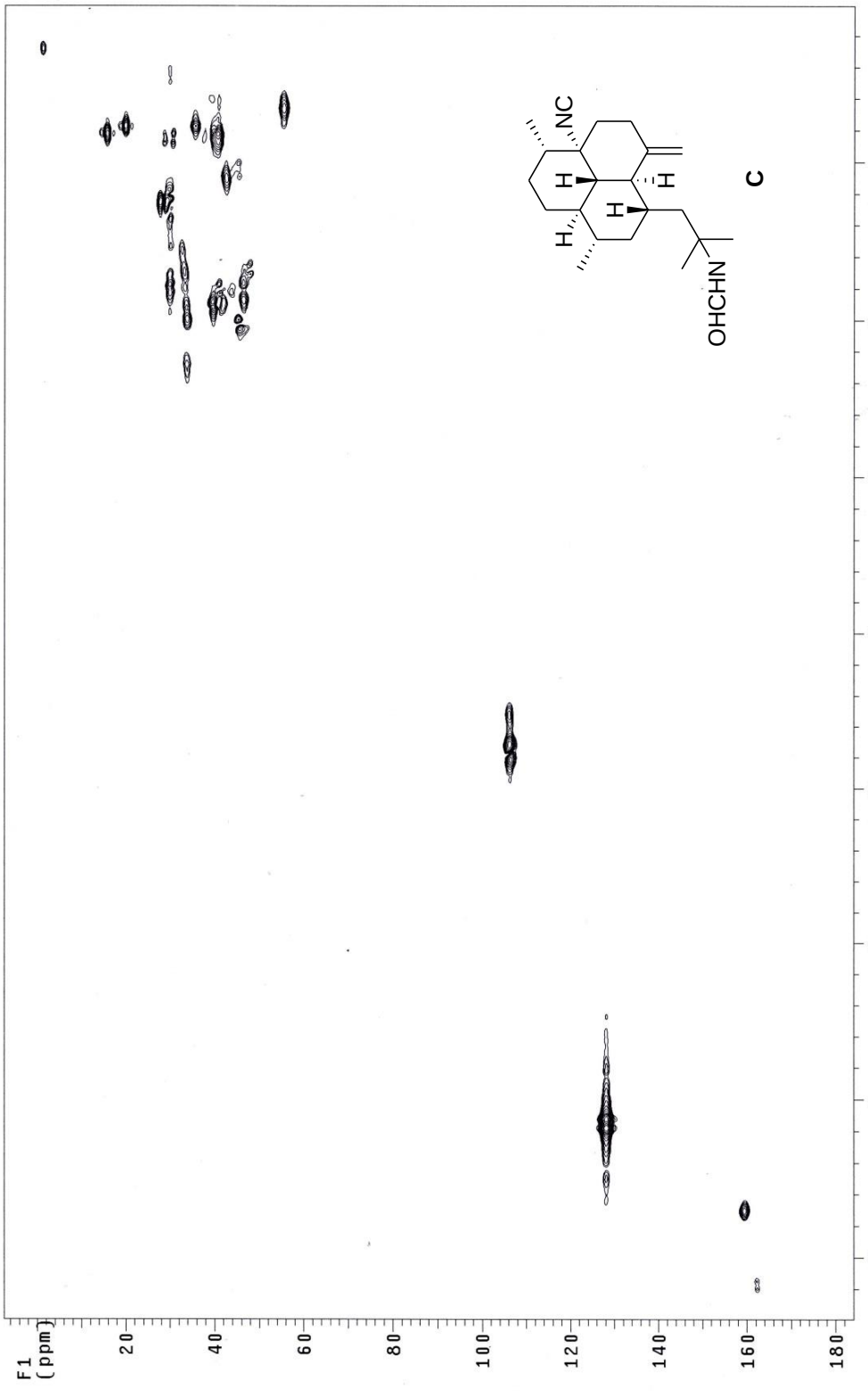
IR spectrum of **C** (CH_2Cl_2)



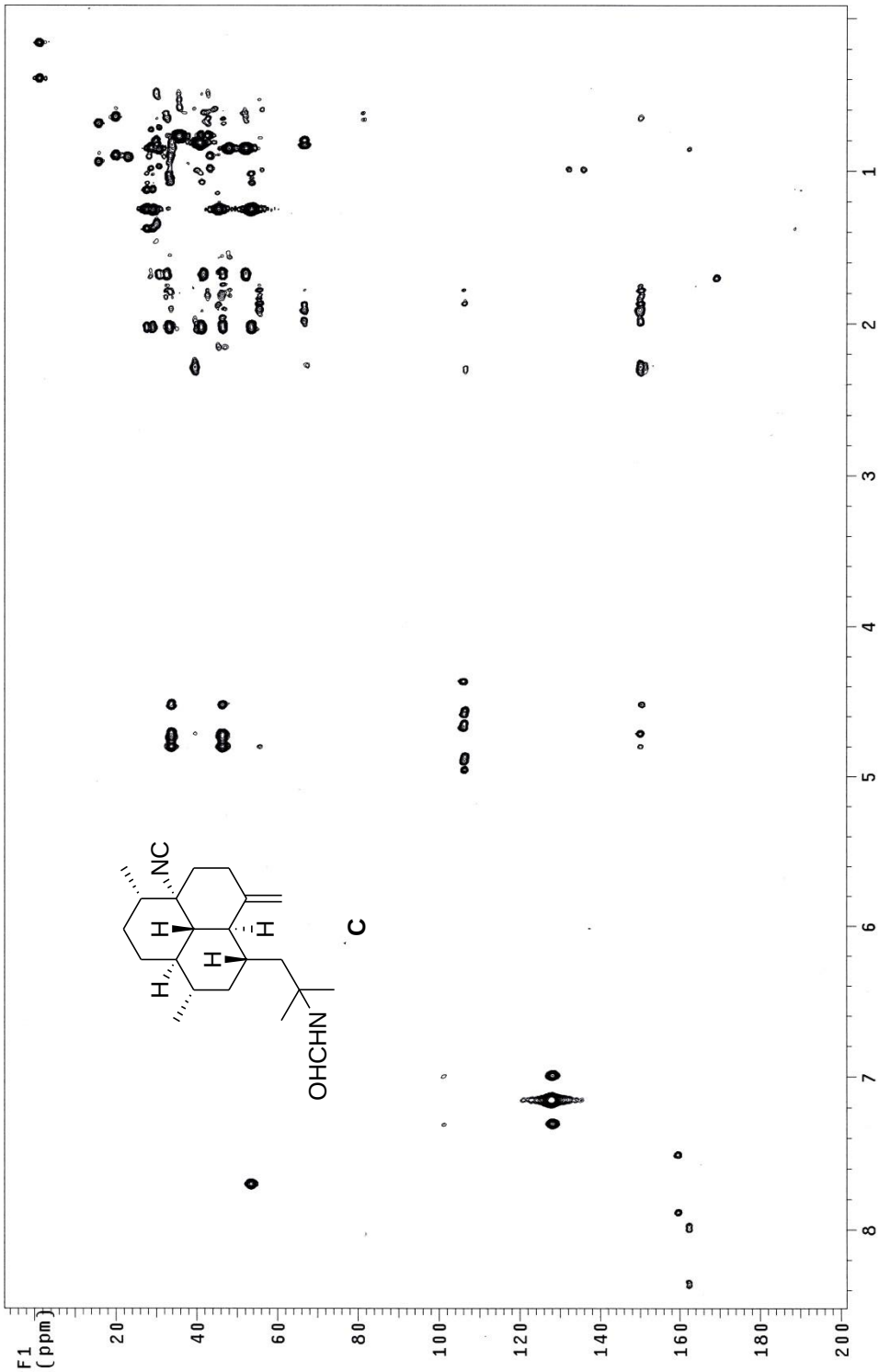
IR spectrum of **C** (thin film)



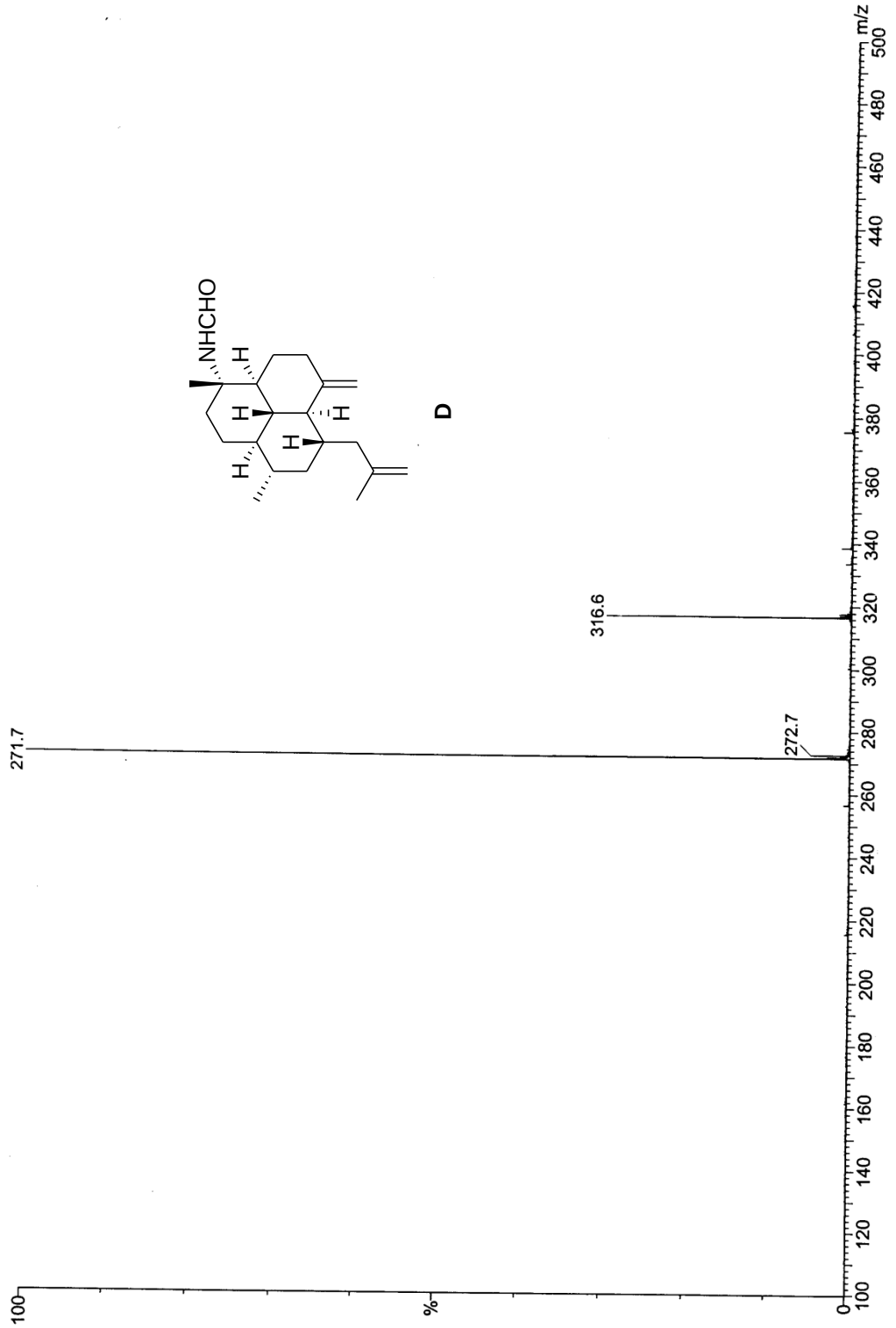
^{1}H, ^{1}H-COSY spectrum of **C** (500 MHz, C_6D_6)



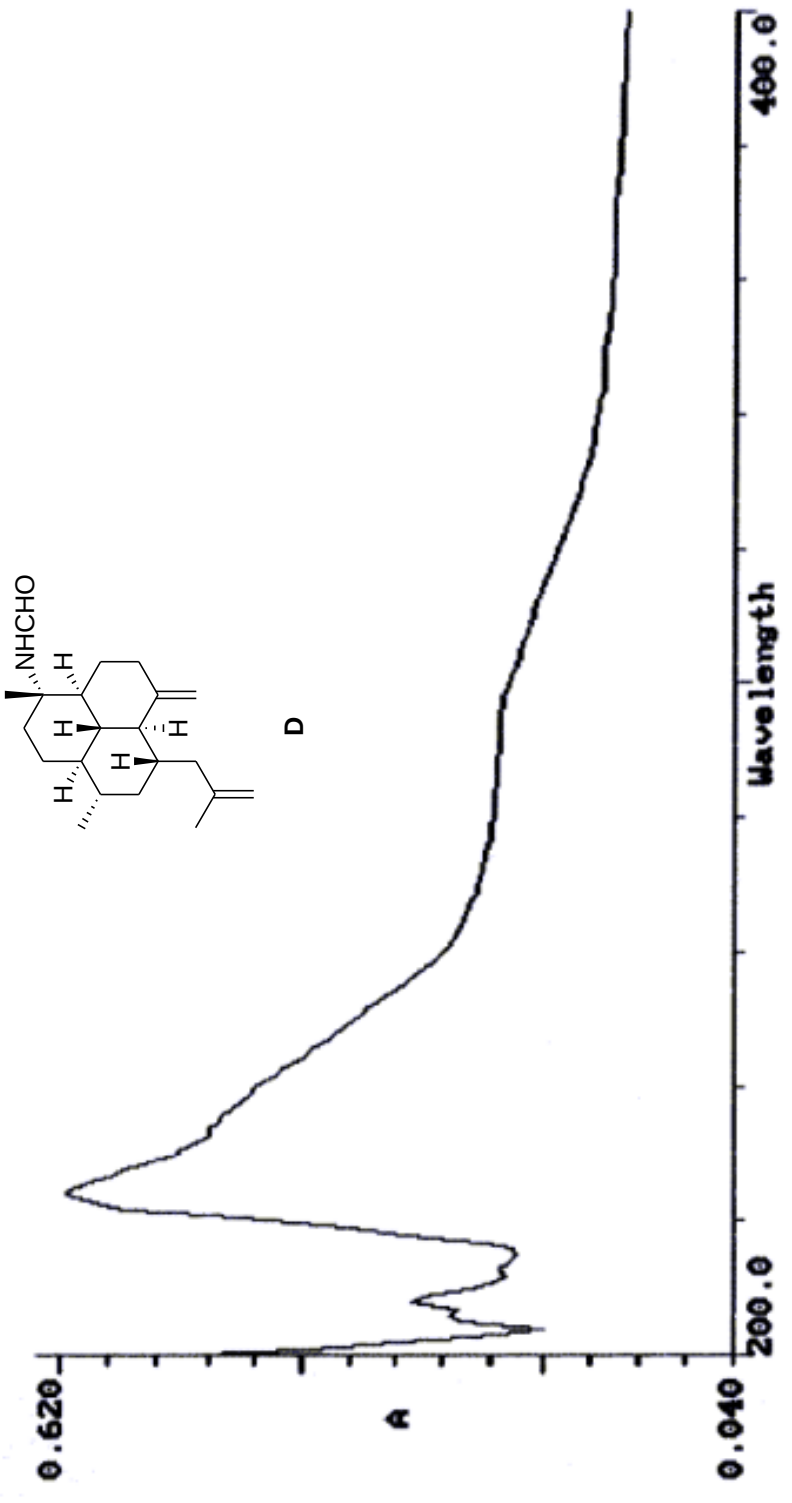
HMQC spectrum of **C** (500 MHz, C_6D_6)



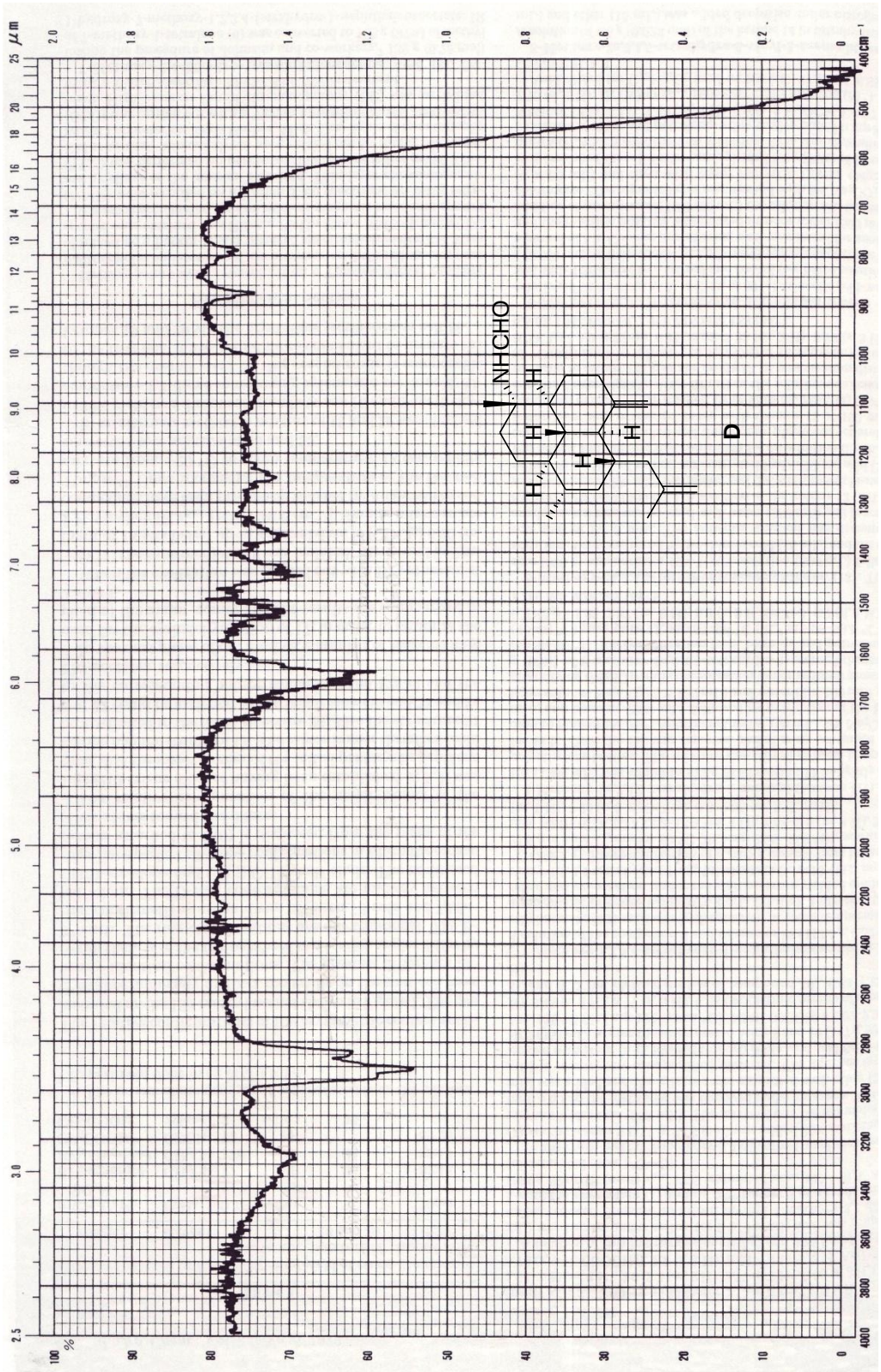
HMBC spectrum of **C** (500 MHz, C_6D_6)



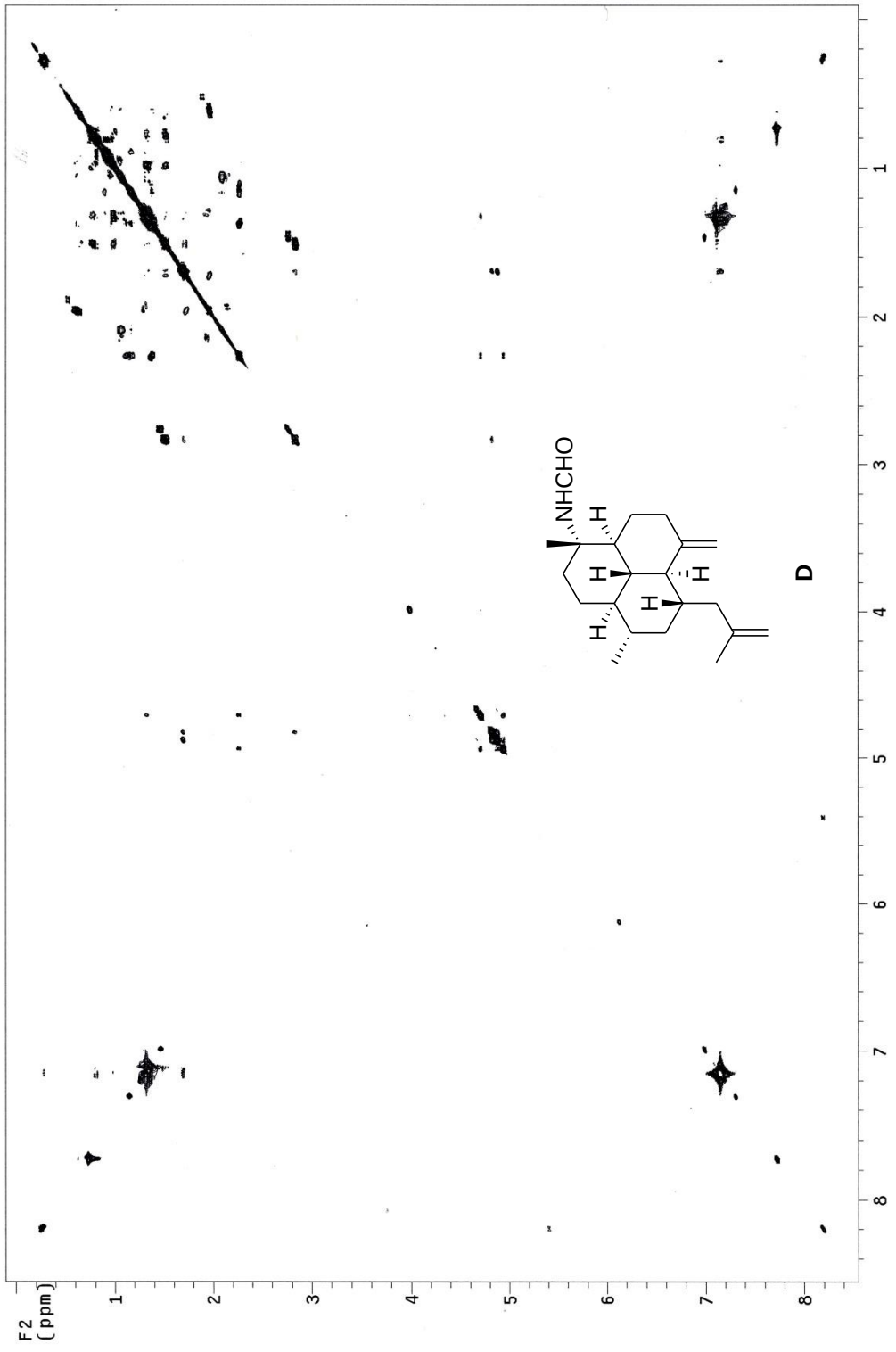
ESIMS spectrum of **D**



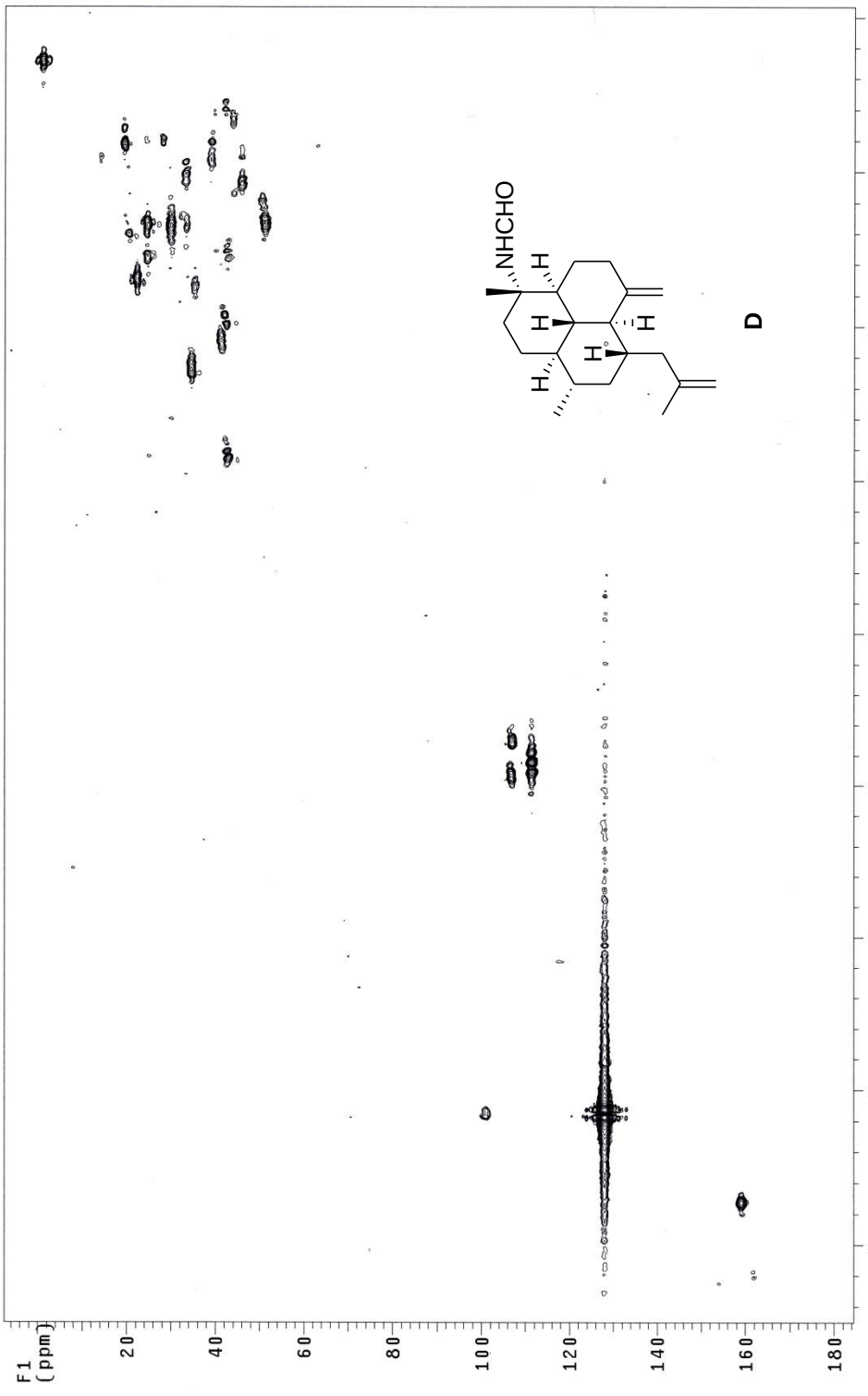
UV spectrum of **D** (CH_2Cl_2)



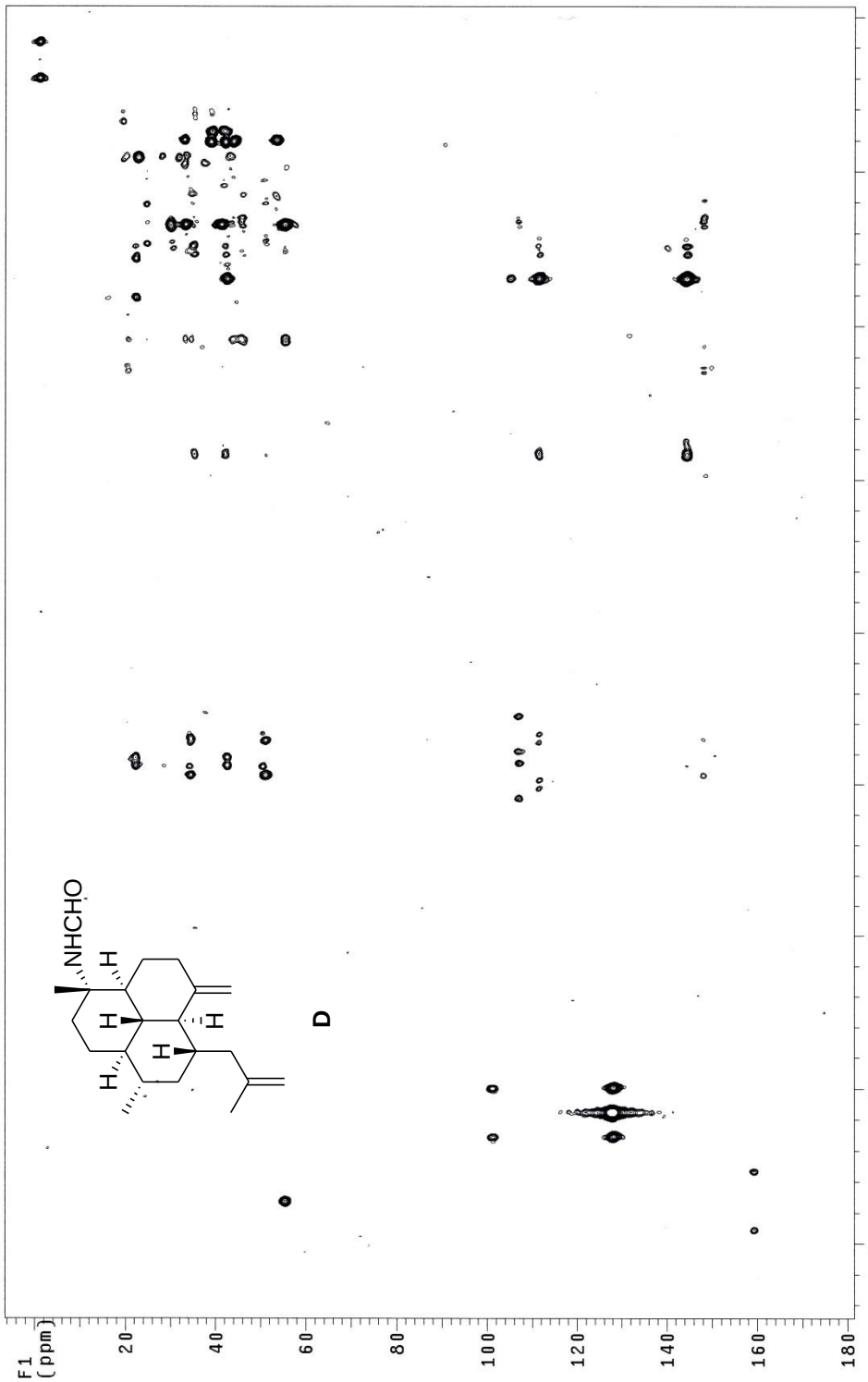
IR spectrum of **D** (thin film)



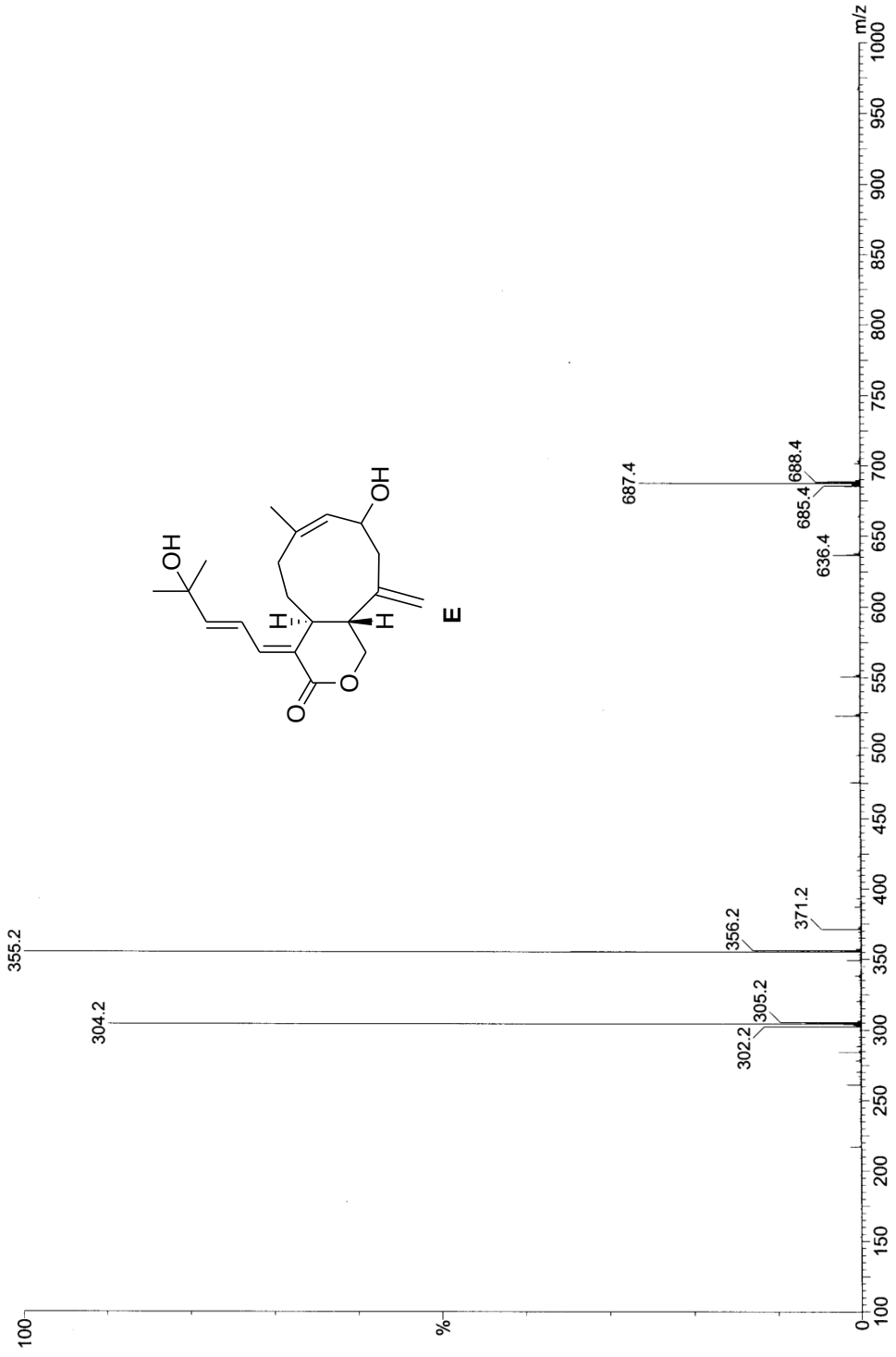
^{1}H, ^{1}H-COSY spectrum of **D** (500 MHz, C_6D_6)



HMQC spectrum of **D** (500 MHz, C_6D_6)



HMBC spectrum of **D** (500 MHz, C_6D_6)



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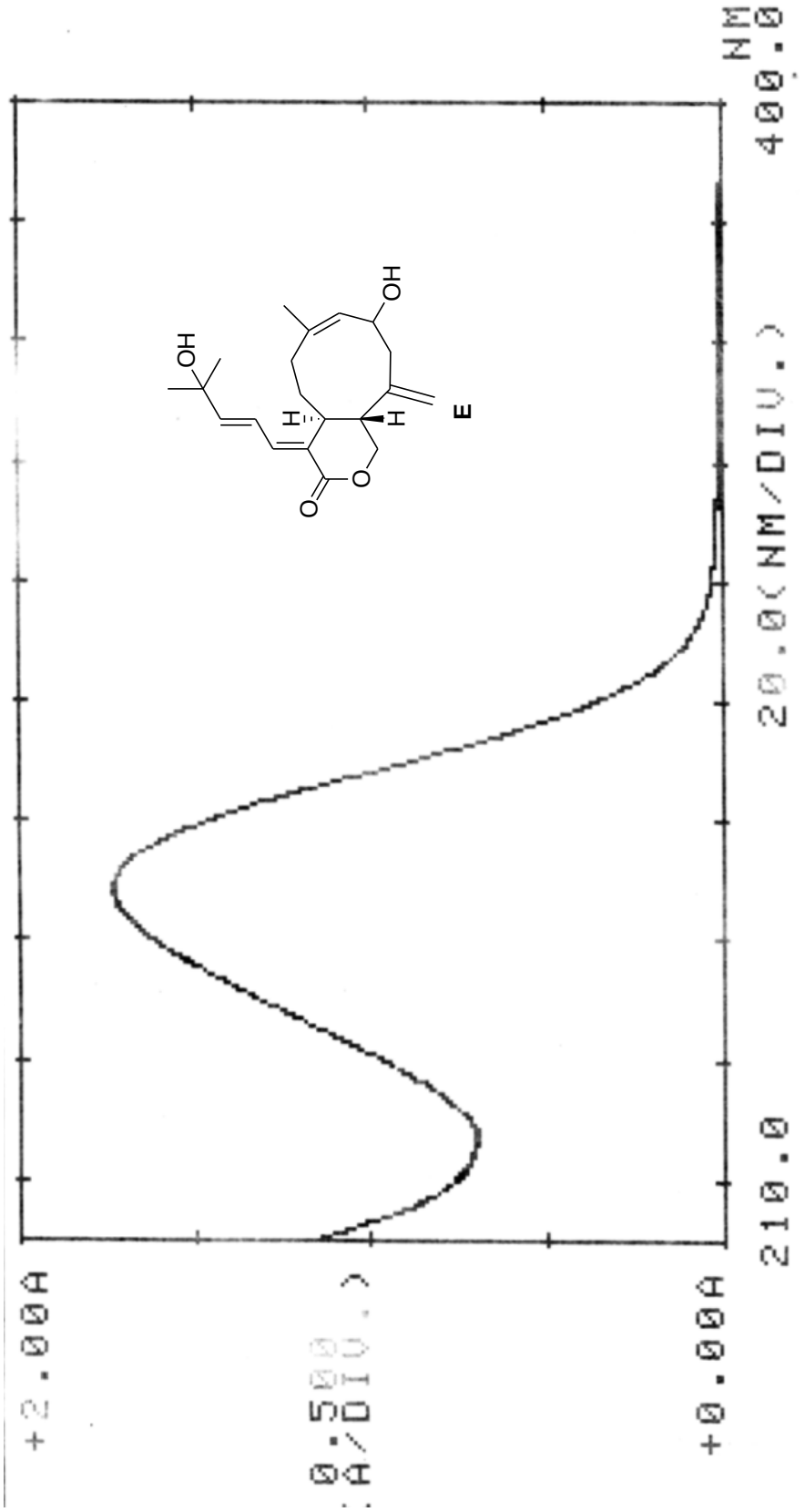
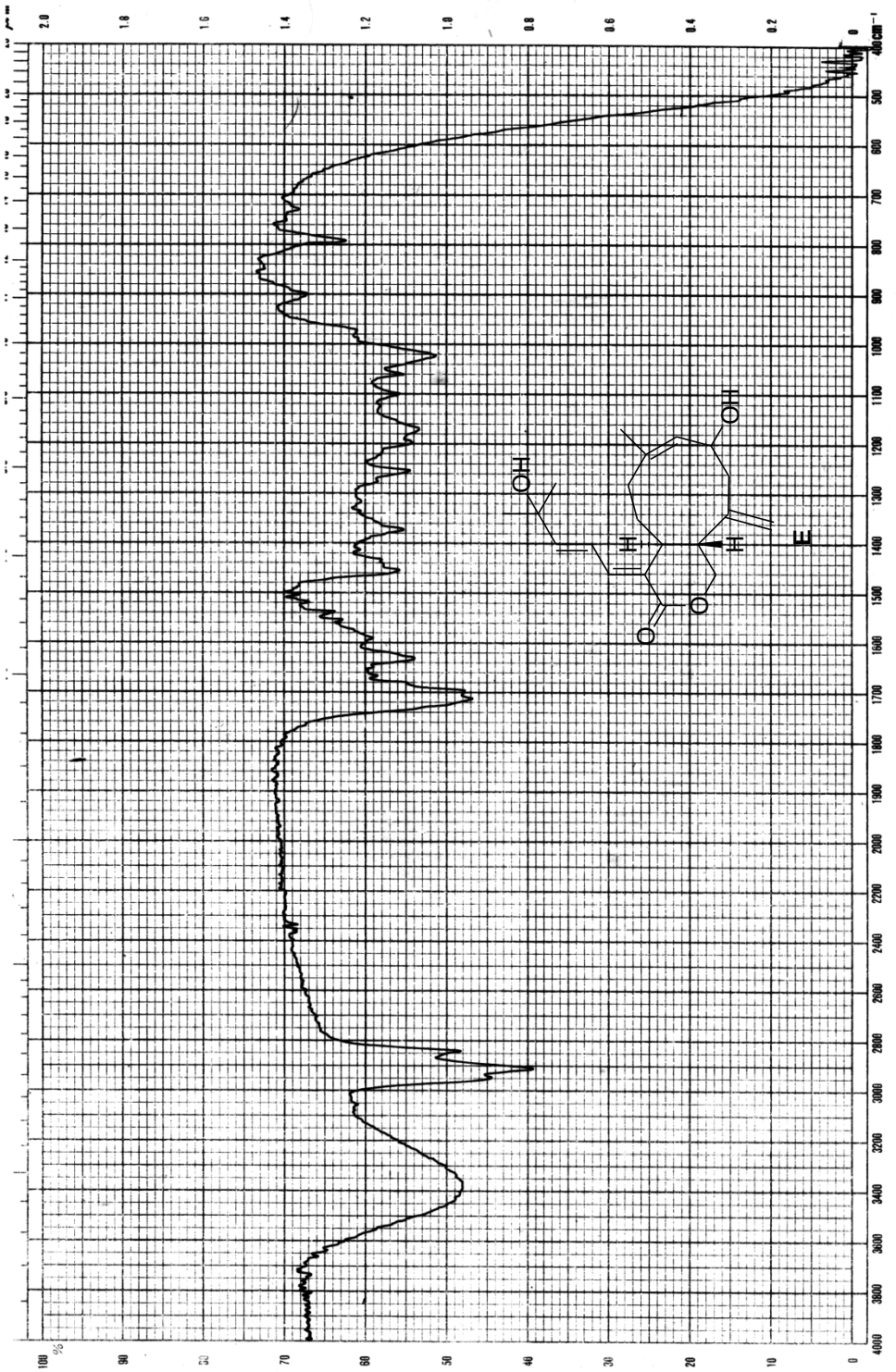
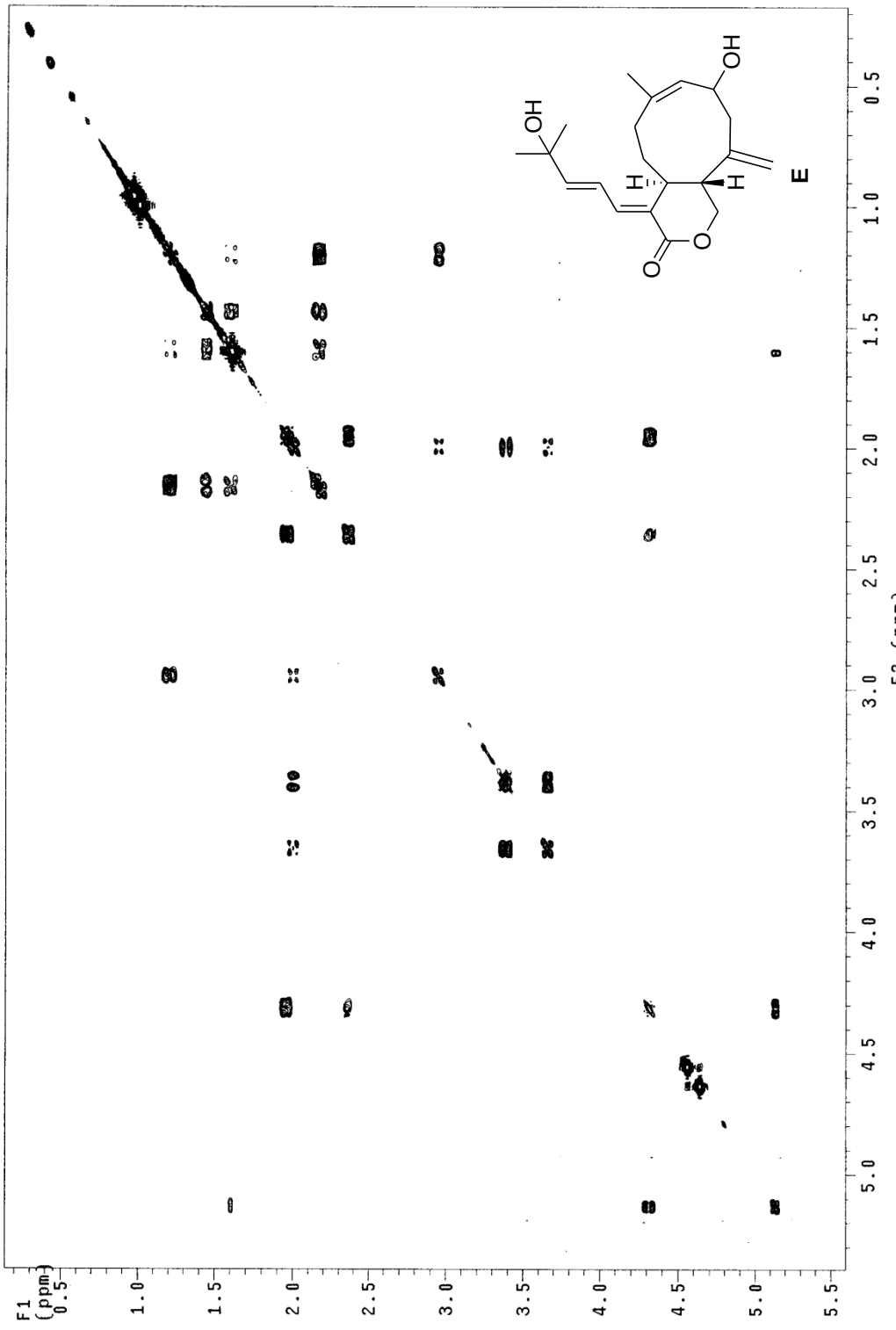


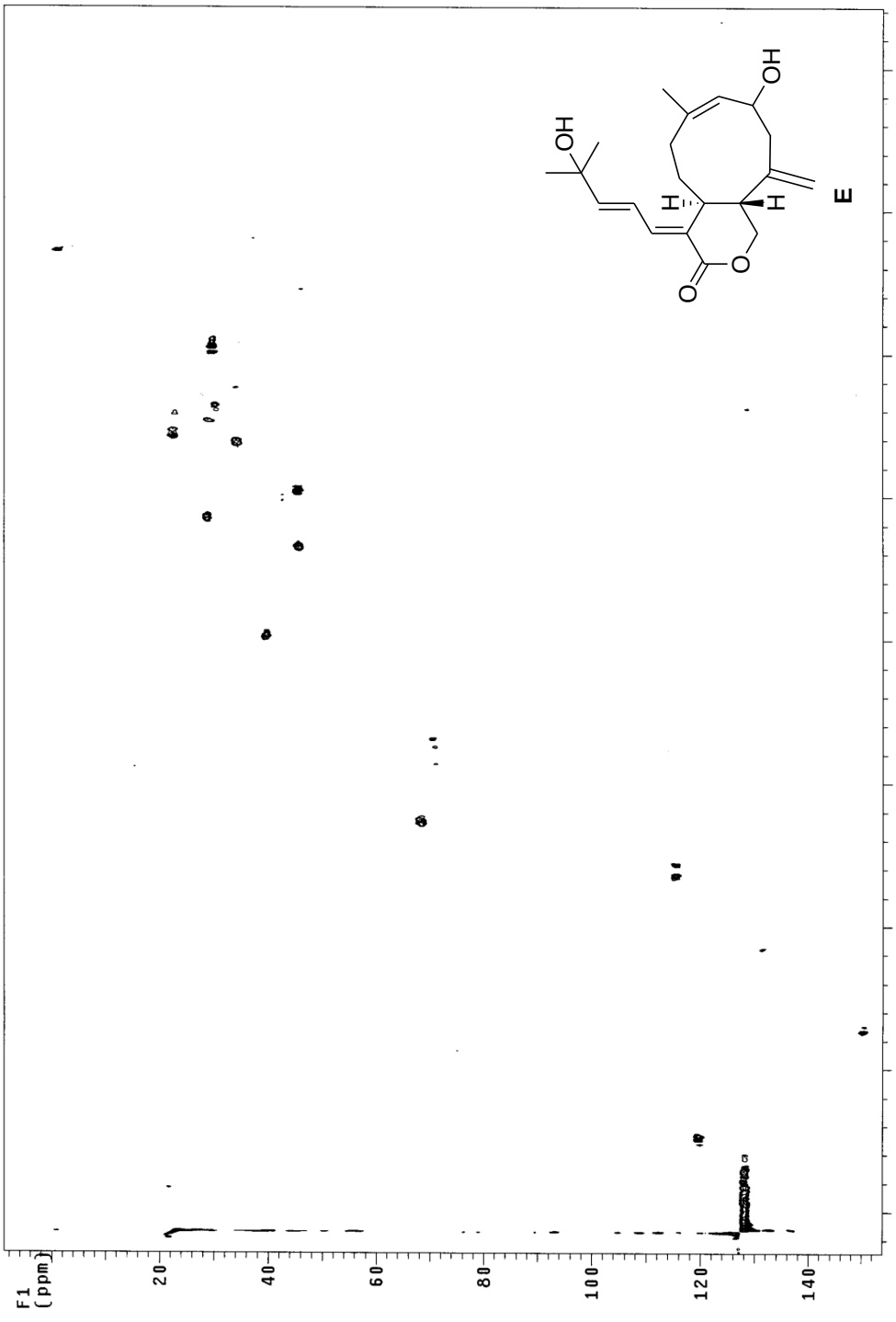
UV spectrum of **E** ($CHCl_3$)



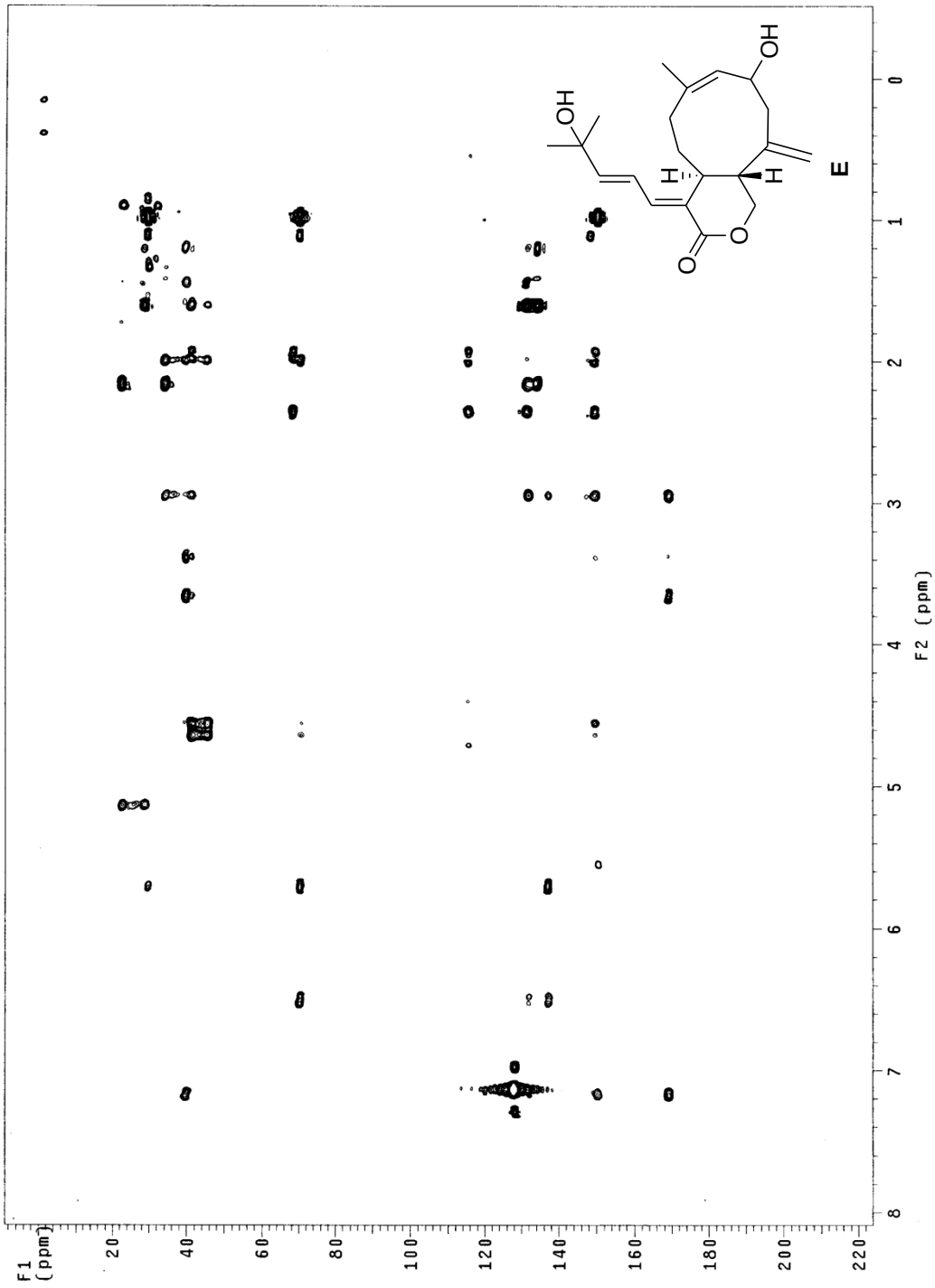
IR spectrum of **E** (thin film)



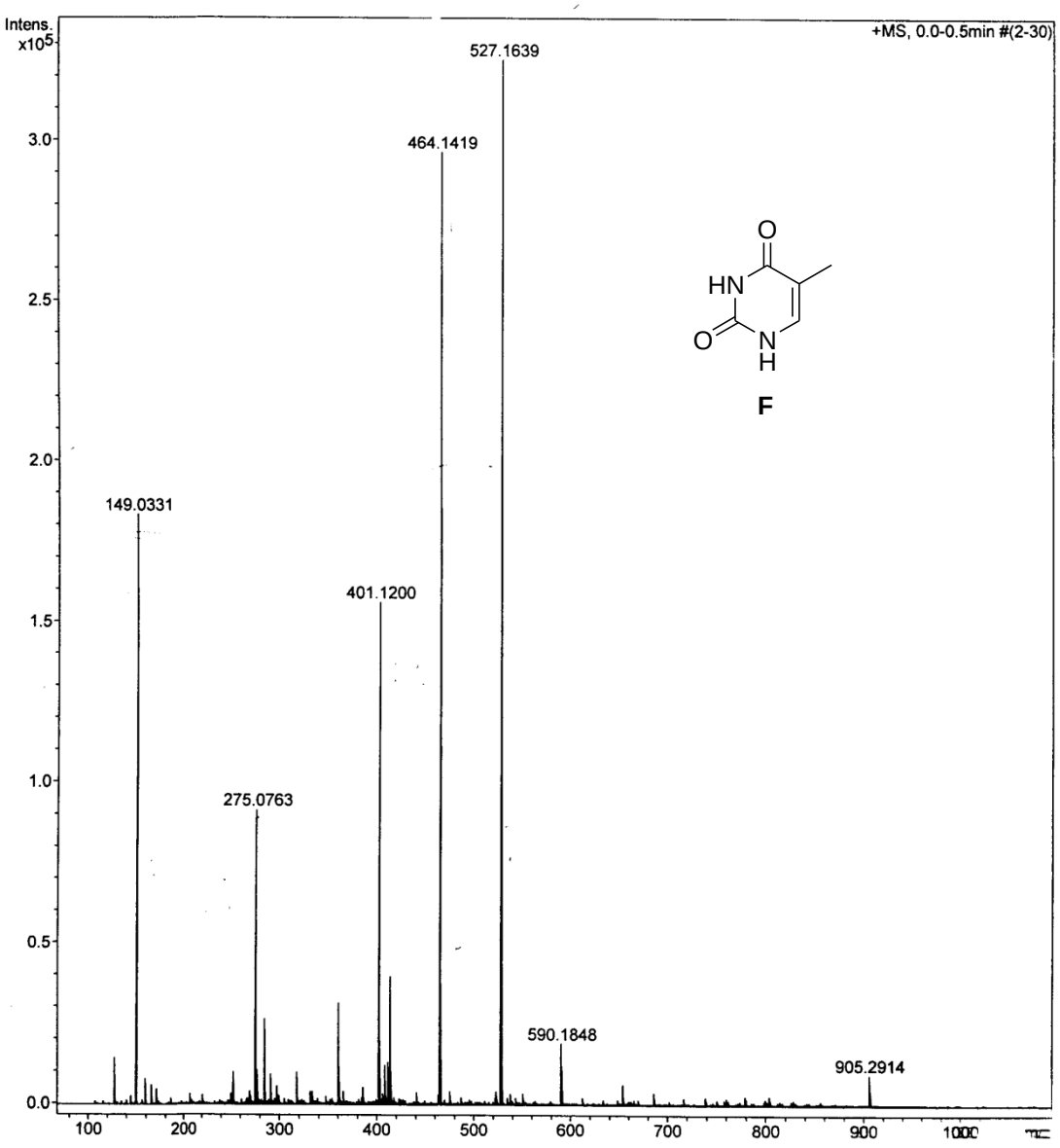
^{1}H, ^{1}H-COSY spectrum of **E** (500 MHz, C_6D_6)



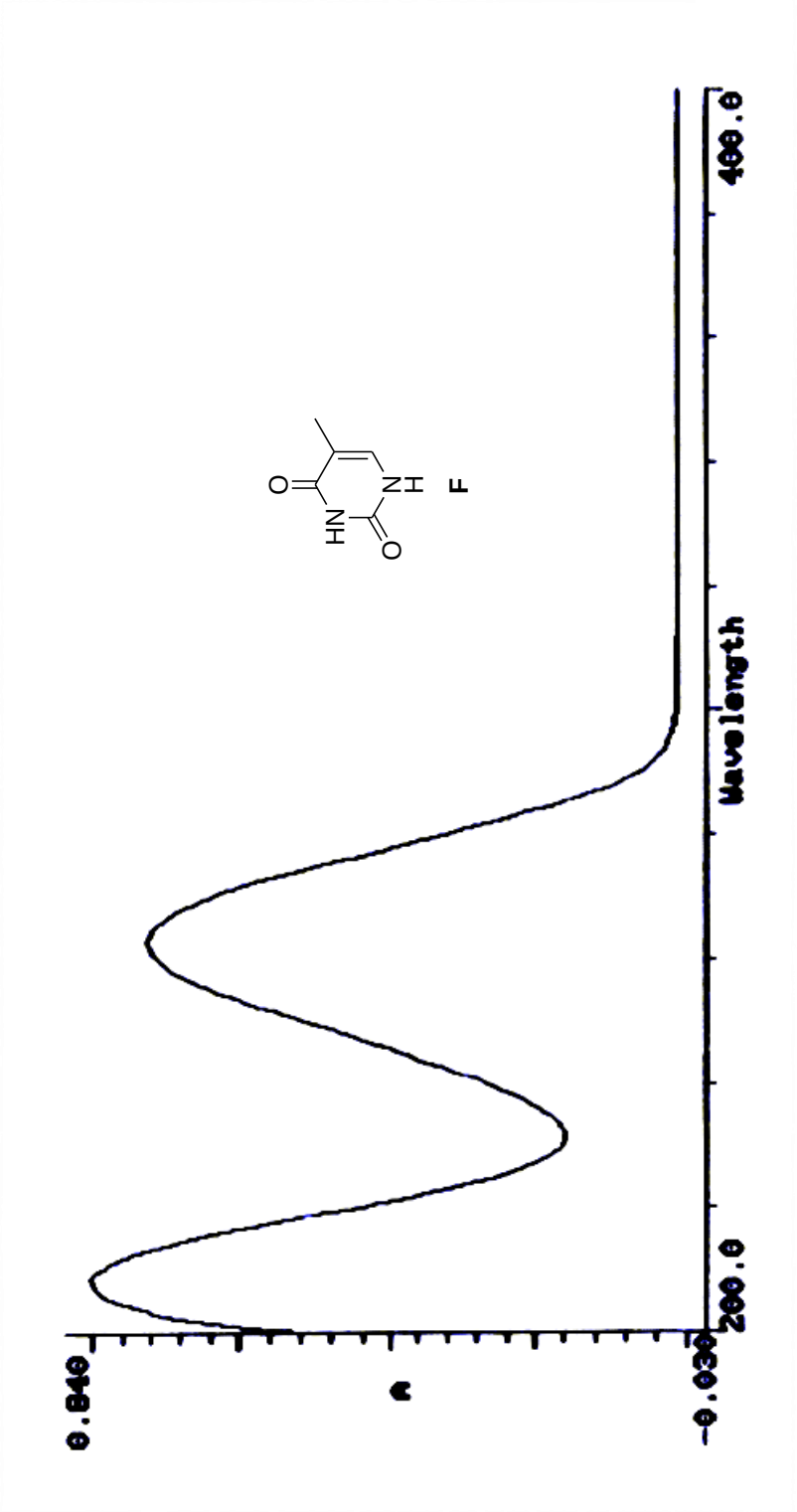
HMQC spectrum of **E** (500 MHz, C_6D_6)



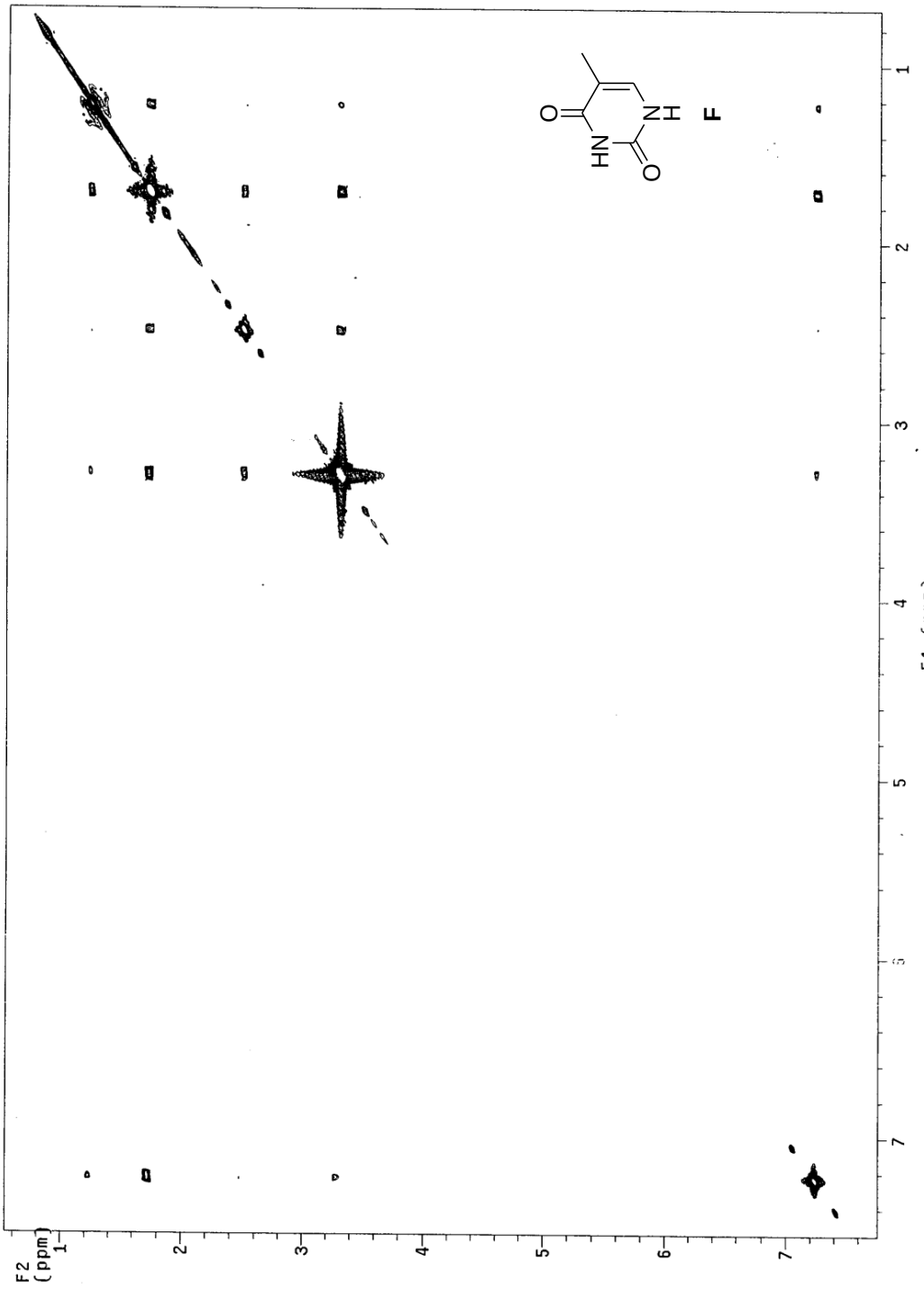
HMBC spectrum of **E** (500 MHz, C_6D_6)



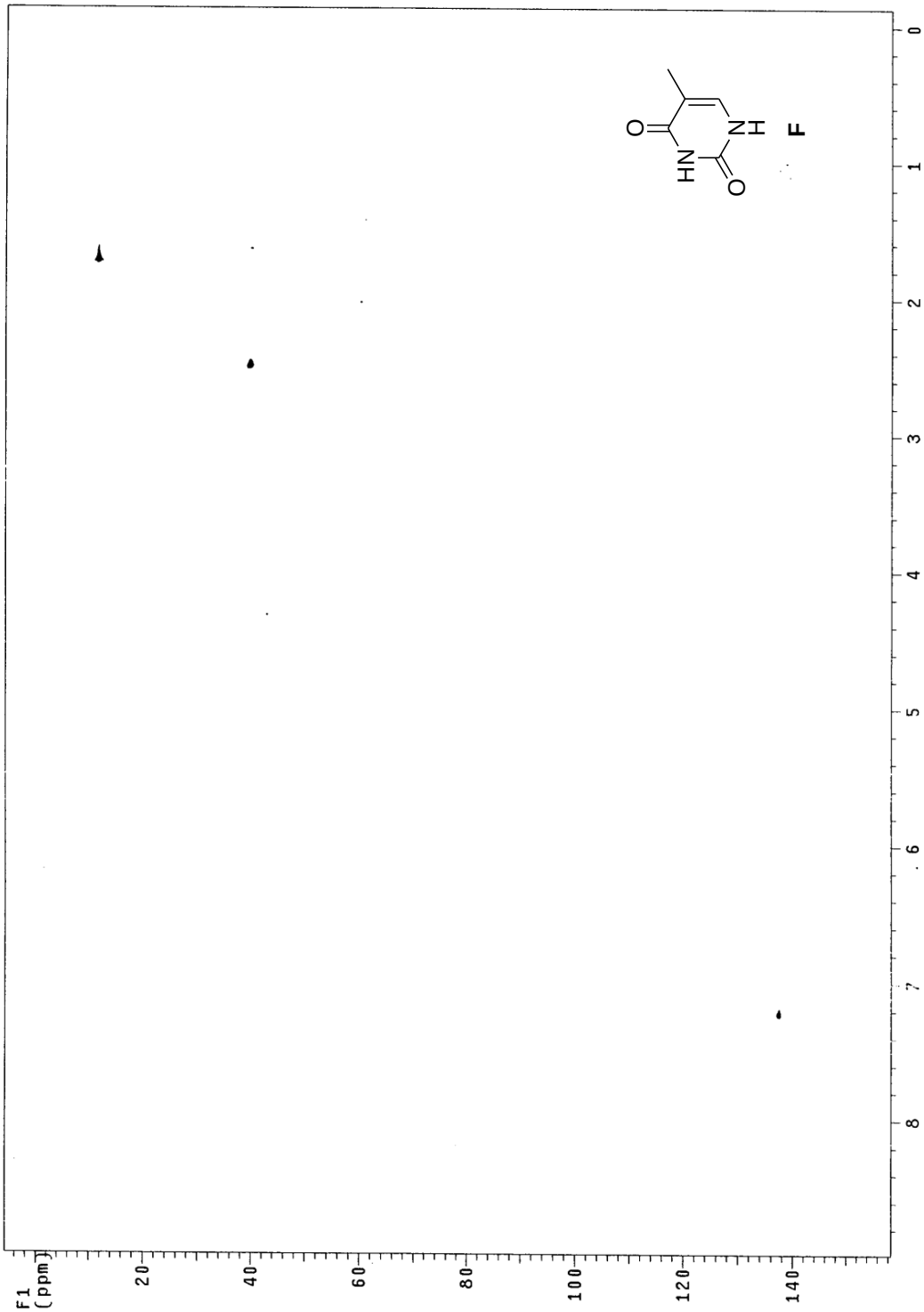
HR-ESIMS spectrum of **F**



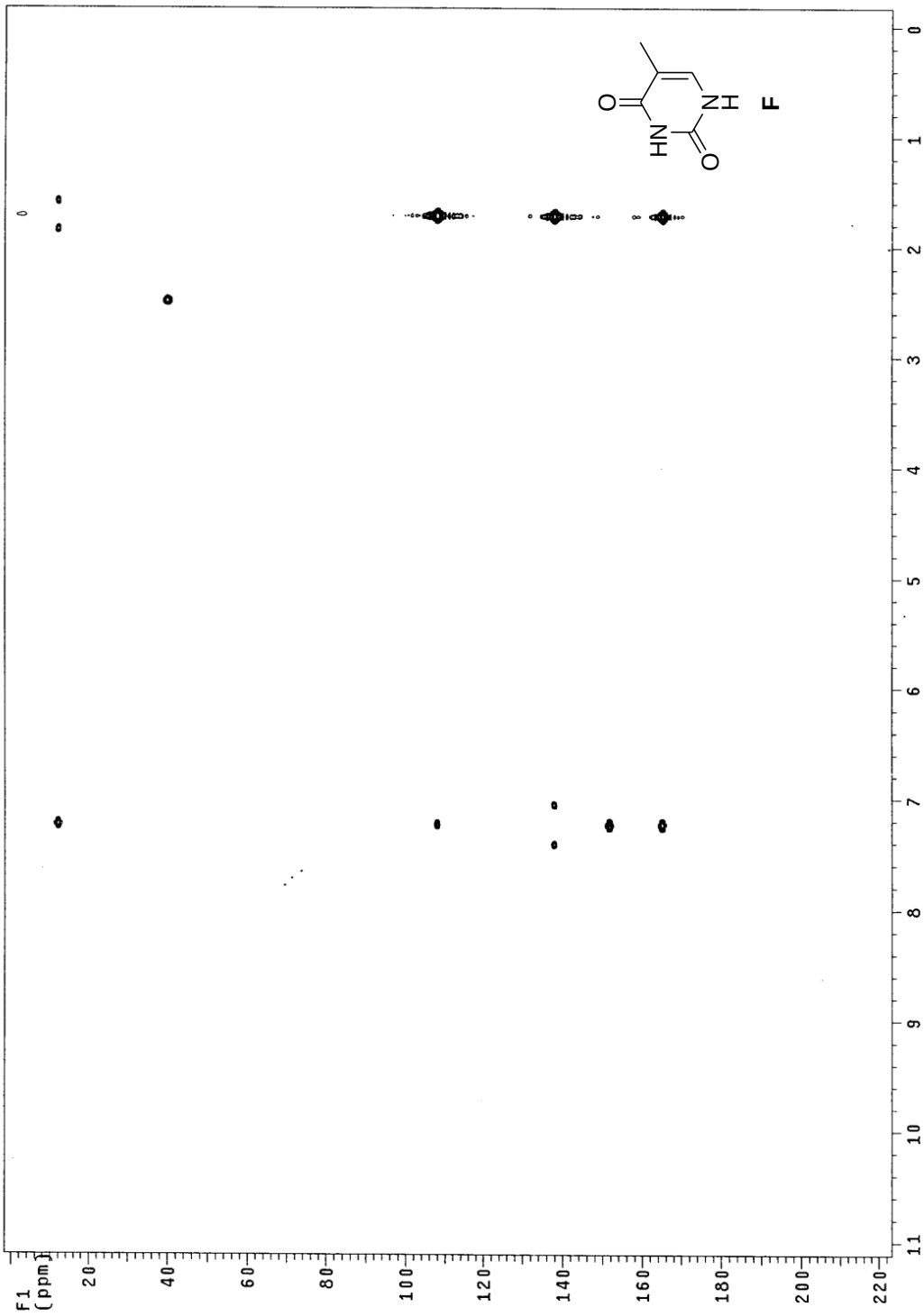
UV spectrum of **F** (MeOH)



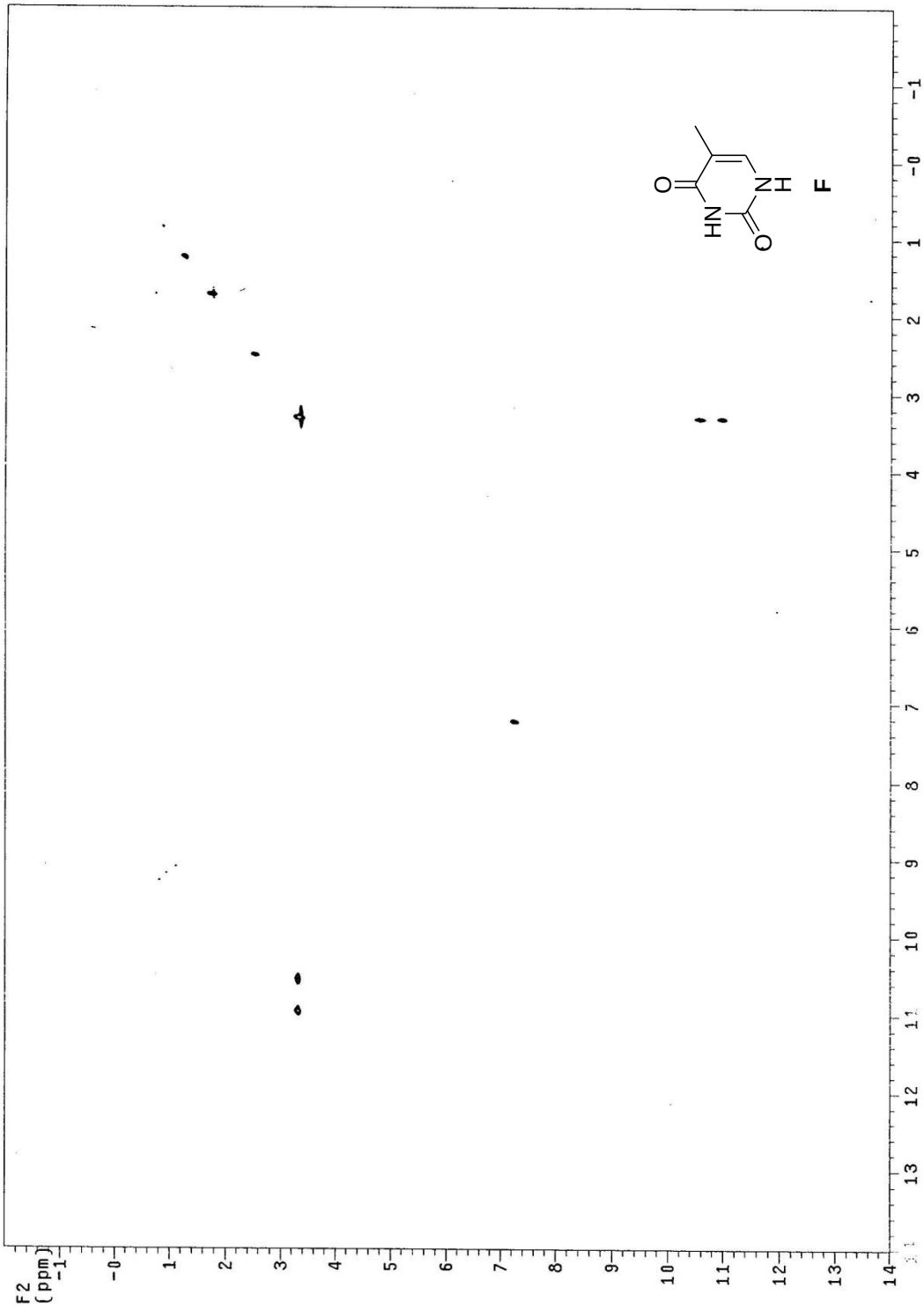
^{1}H, ^{1}H-COSY spectrum of **F** (500 MHz, DMSO-d_6)



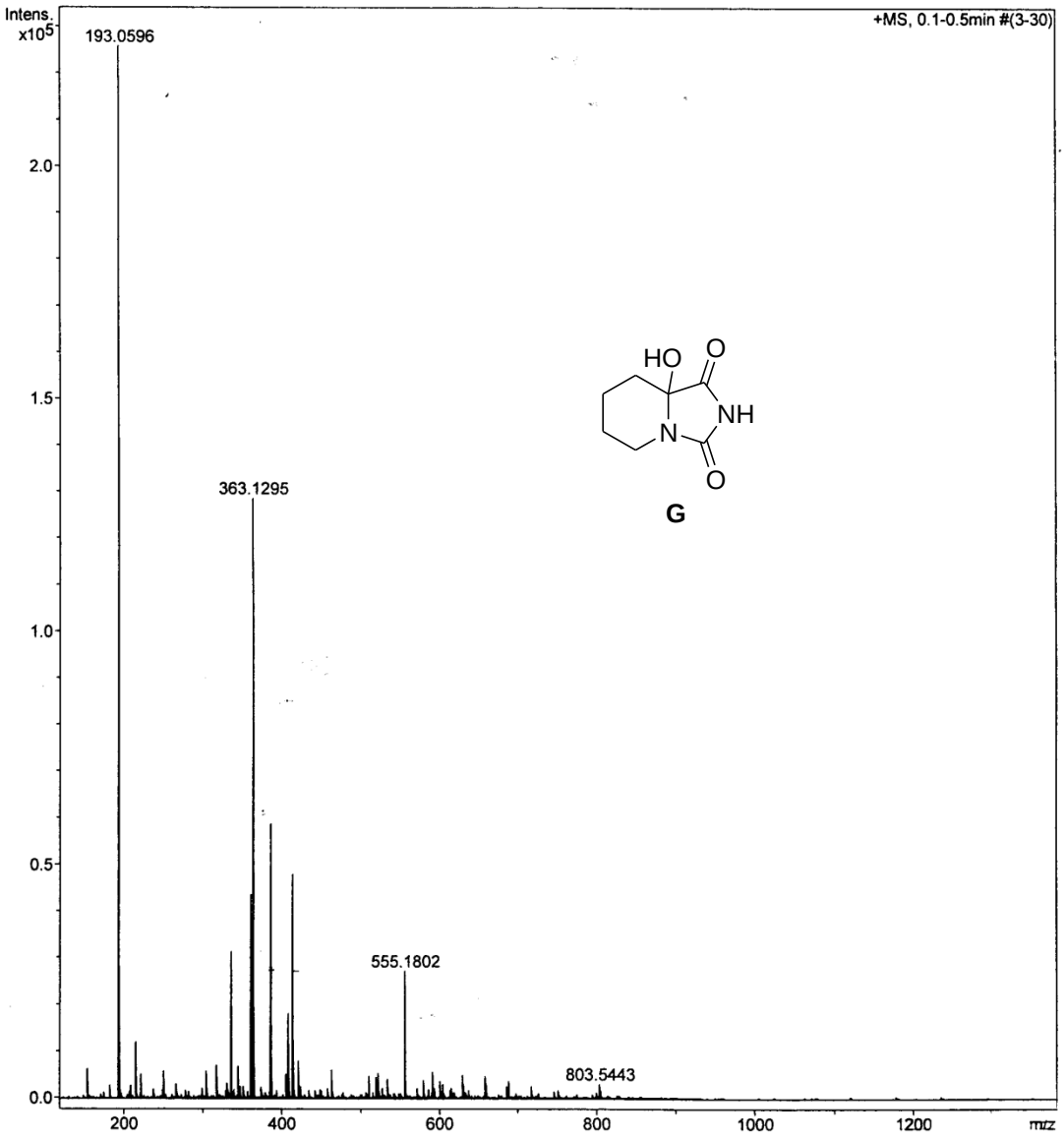
HMQC spectrum of **F** (500 MHz, DMSO-d_6)



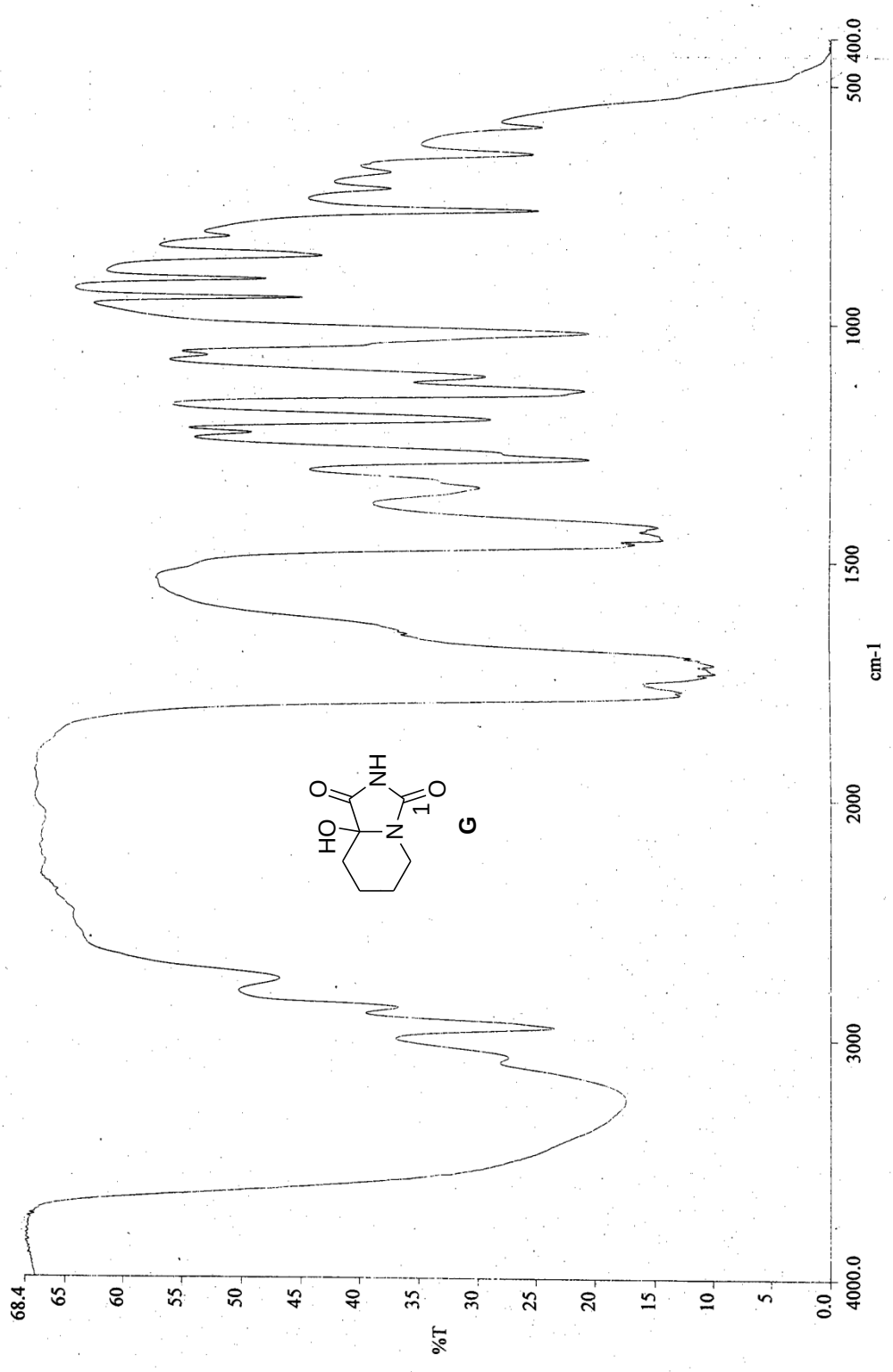
HMBC spectrum of **F** (500 MHz, DMSO-d_6)



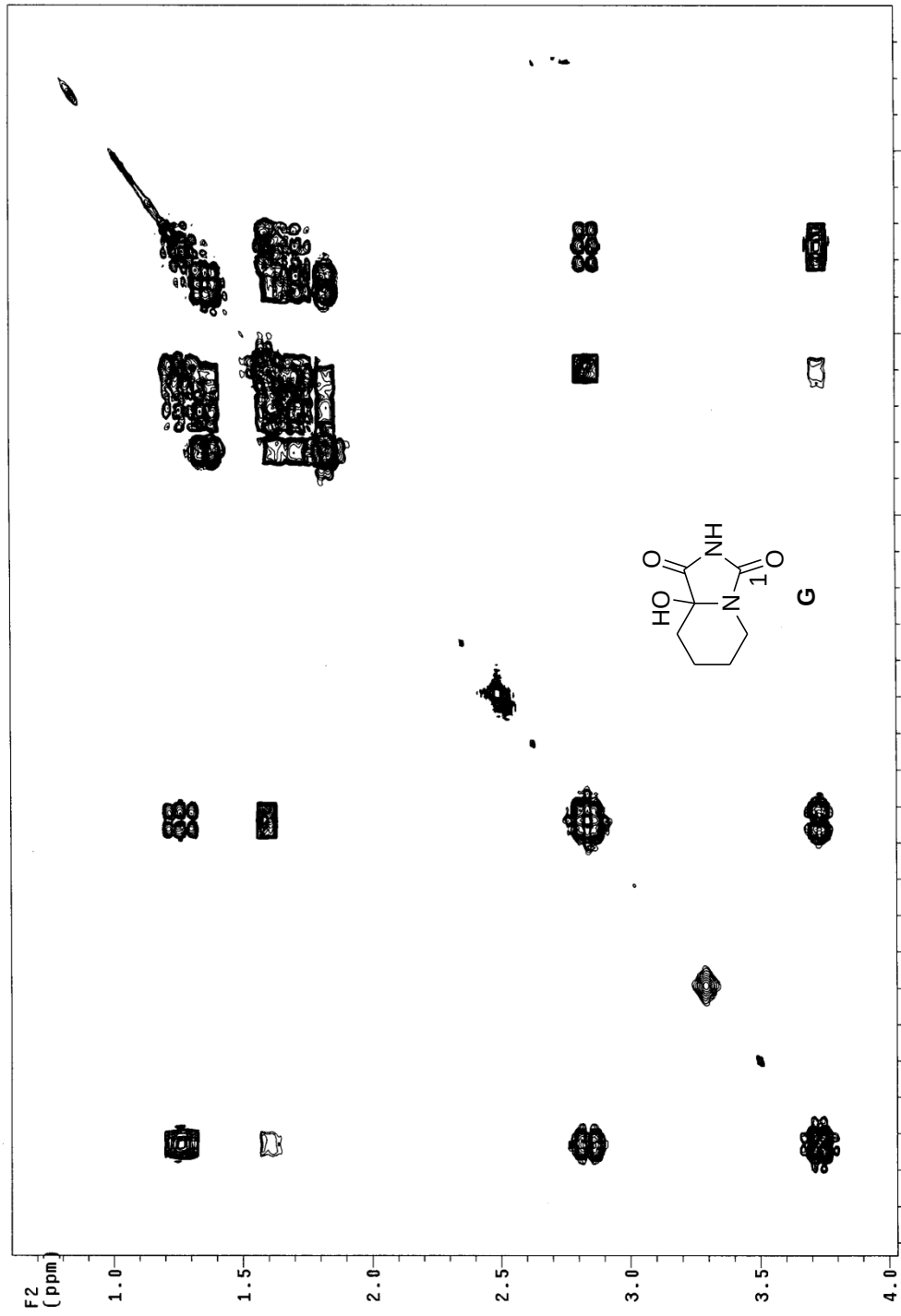
NOESY spectrum of **F** (500 MHz, DMSO-d_6)



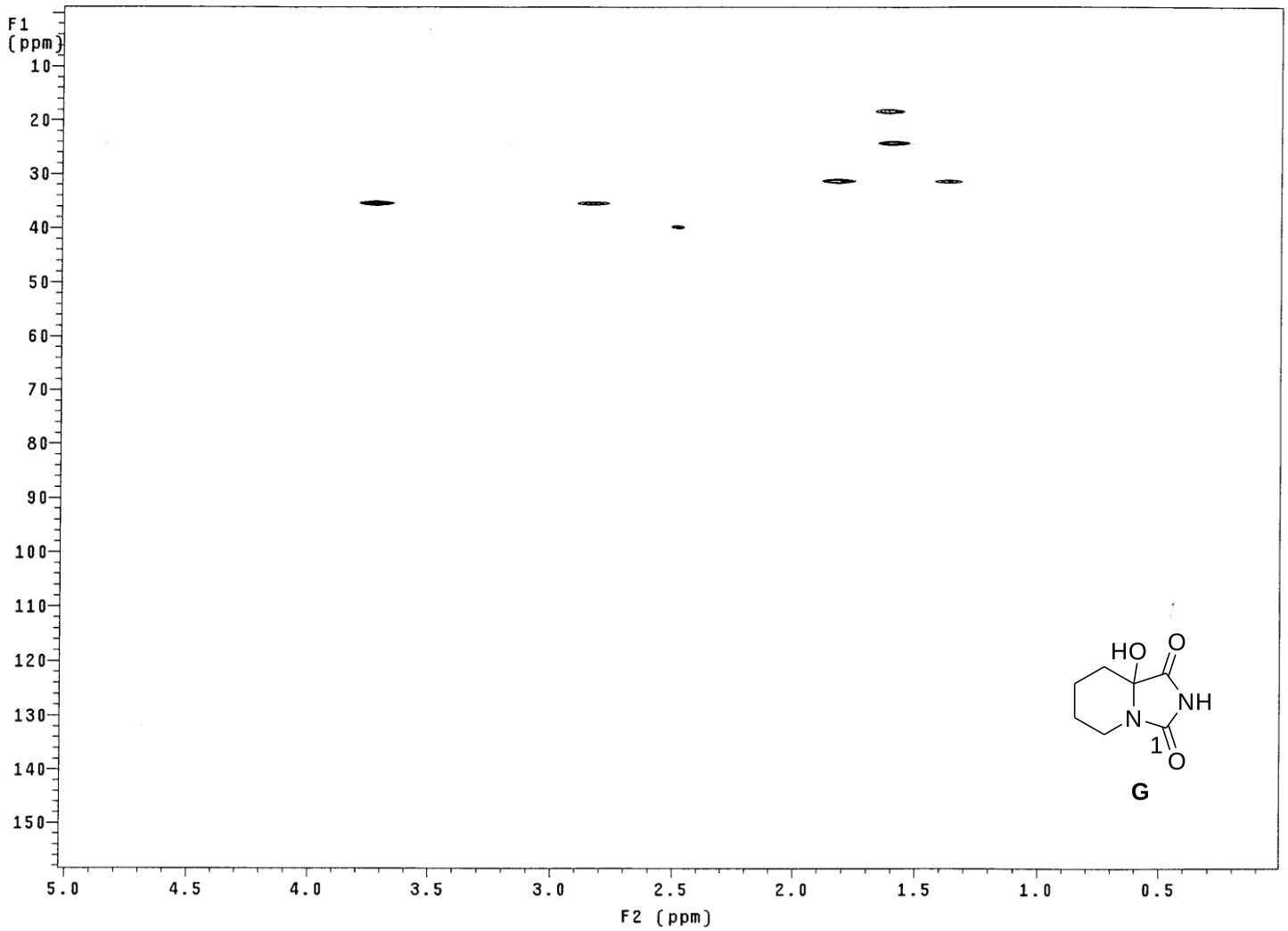
HR-ESIMS spectrum of **G**



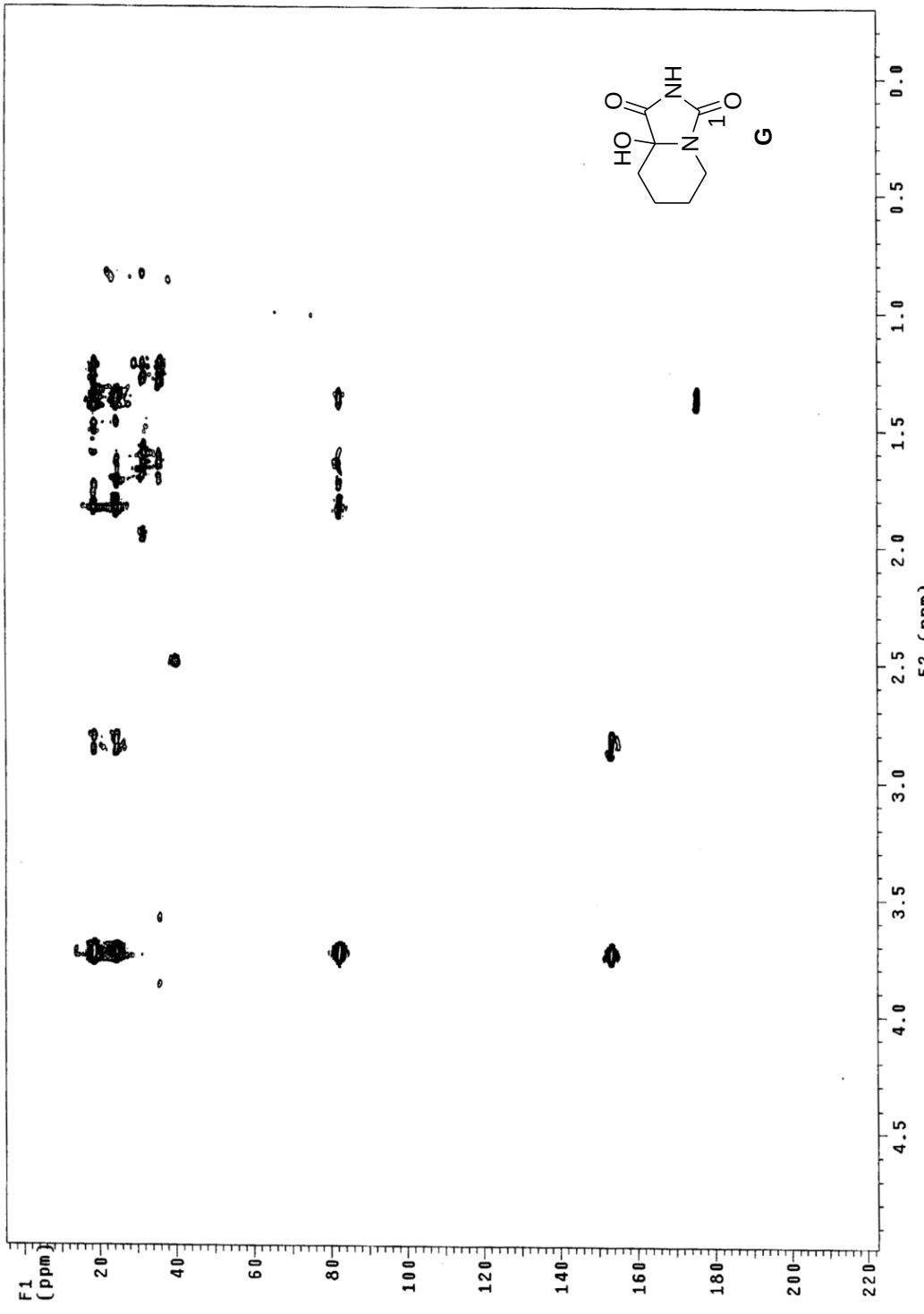
IR spectrum of **G** (thin film)



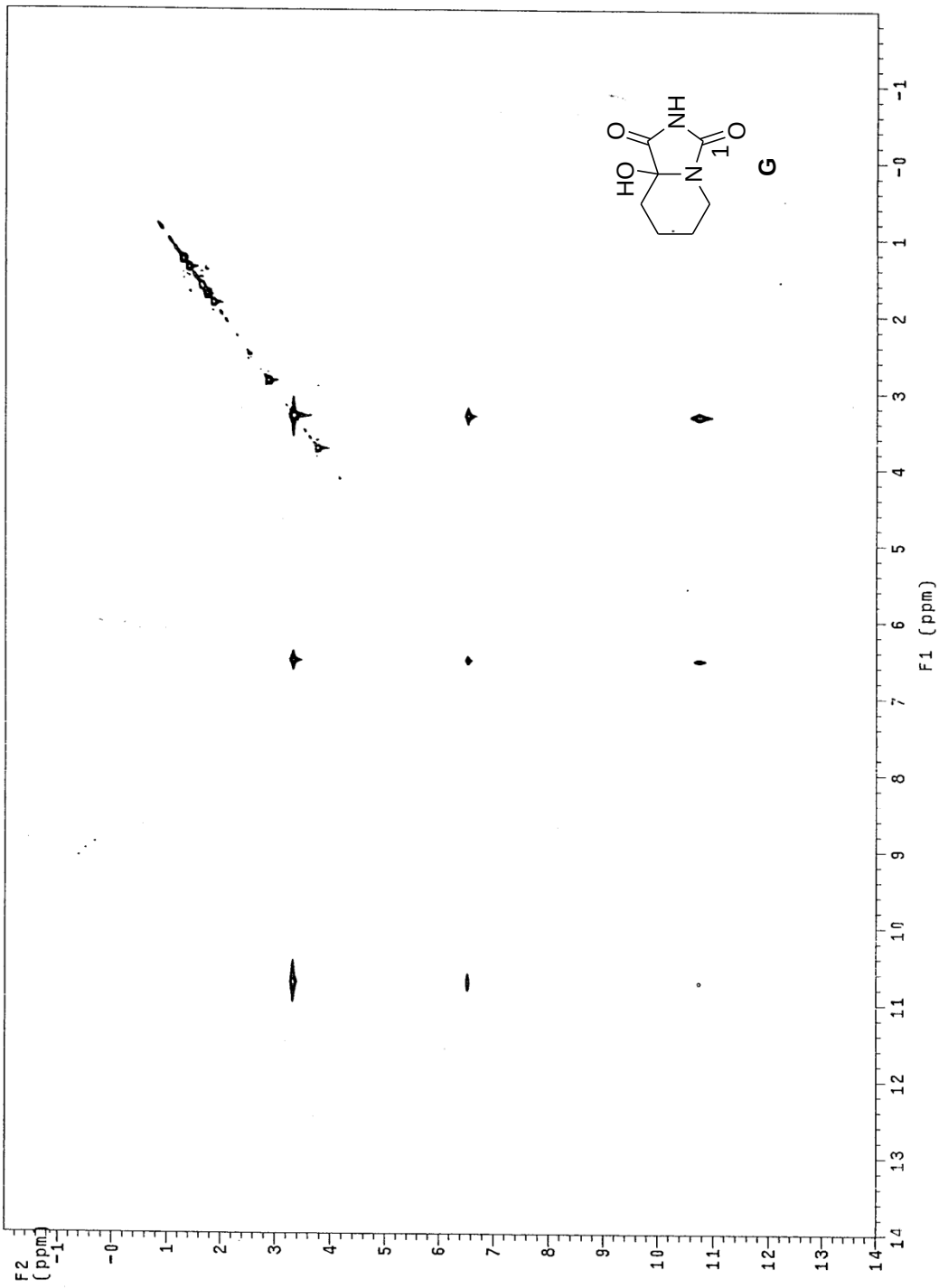
^{1}H, ^{1}H-COSY spectrum of **G** (500 MHz, DMSO-d_6)



HMQC spectrum of **G** (500 MHz, DMSO-d_6)



HMBC spectrum of **G** (500 MHz, DMSO-d_6)



NOESY spectrum of **G** (500 MHz, DMSO-d_6)

VITAE

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List of Publication and Proceeding

Wattanapiromsakul, C.; Chanthathamrongsiri, N.; Bussarawit, S.; Yuenyongsawad, S.; Plubrukarn, A.; Suwanborirux, K. 8-Isocyanoamphilecta-11(20),15-diene, a new anti-malarial isonitrile diterpene from the sponge *Ciocalapata* sp. *Can. J. Chem*. **2009**. *87*, 612 - 618.

Chanthathamrongsiri, N.; Yuenyongsawad, S.; Wattanapiromsakul, C.; Plubrukarn, A. Bifunctionalized Amphilectane Diterpenes from the Sponge *Stylissa* cf. *massa*. *J. Nat. Prod*. **2012**, *75*, 789 - 792.