Cytotoxic Compounds from Marine Gliding Bacteria in Thailand

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ชื่อวิทยานิพนธ์ สารออกฤทธิ์ต่อเซลล์จากไกลดิงแบคทีเรียในทะเลของประเทศไทย
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บทคัดย่อ

แบคทีเรียไกลดิงทั้งหมด 27 สายพันธุ์ แยกได้จากตัวอย่างชนิดต่างๆ ที่เก็บในทะเลบริเวณอ่าวไทย โดยพบว่าฟิล์มชีวภาพเป็นแหล่งที่ดีที่สุดในการแยกเชื้อชนิดนี้ รองลงมาคือ ฟองน้ำ ซากพืช สาหร่าย และกระดองปู ตามลำดับ นอกจากนี้ยังพบว่าเทคนิค subculture และการใช้ micromanipulator เป็นเทคนิคที่เหมาะสมในการแยก และทำบริสุทธิ์เชื้อกลุ่มนี้ จากการวิเคราะห์ลำดับนิวคลีโอไทด์ของชิ้นยีนส์ 16S rRNA พบว่าแบคทีเรียไกลดิงที่ใช้ในการศึกษาสามารถจำแนกได้เป็น 6 กลุ่ม ซึ่งทั้งหมดเป็นสมาชิกในกลุ่มเชื้อ *Cytophaga-Flavobacterium-Bacteriodes* (CFB) โดยแบคทีเรียไกลดิงในกลุ่มที่ 1, 4, 5 และ 6 มีความเป็นไปได้สูงที่จะเป็นแบคทีเรียไกลดิงสกุลใหม่ โดยที่แบคทีเรียไกลดิงในกลุ่มที่ 4, 5 และ 6 นั้นได้รับการจัดจำแนกเป็นแบคทีเรียไกลดิงสกุลใหม่เรียบร้อยแล้ว คือ *Rapidithrix thailandica* gen. nov., sp. nov., *Aureispira marina* gen. nov., sp. nov. และ *Aureispira maritima* sp. nov. ตามลำดับ สำหรับแบคทีเรียไกลดิงในกลุ่ม 2 นั้นมีความเหมือนกับ *Tenacibaculum mesophilum* ในขณะที่แบคทีเรียไกลดิงในกลุ่ม 3 อาจเป็นไปได้ที่จะเป็นสปีชีส์ใหม่ของ *Fulvivirga kasyanovii* และจากการศึกษาความสามารถในการผลิตสารออกฤทธิ์ทางชีวภาพในการยับยั้งเซลล์มะเร็ง 4 ชนิด ซึ่งประกอบด้วย มะเร็งปากมดลูก (cervical cancer, HeLa) มะเร็งลำไส้ (colon cancer, HT-29) มะเร็งช่องปาก (oral cancer, KB) และมะเร็งเต้านม (breast adenocarcinoma, MCF-7) ของแบคทีเรียไกลดิงที่แยกได้ทั้งจากงานวิจัยนี้ และที่มีอยู่แล้วในคลังสายพันธุ์เชื้อ พบว่าแบคทีเรียไกลดิงในกลุ่มที่ 2 และ 4 มีความสามารถในการผลิตสารที่มีฤทธิ์ยับยั้งเซลล์มะเร็งได้ดีทั้ง 4 ชนิด ส่วนแบคทีเรียไกลดิงในกลุ่มที่ 6 และกลุ่มที่ไม่สามารถจัดจำแนกได้ สามารถผลิตสารที่มีฤทธิ์ยับยั้งเซลล์มะเร็งได้เพียง 2 ชนิด คือ HeLa และ HT-29 ตามลำดับ ซึ่งต่างจากแบคทีเรียไกลดิงในกลุ่มที่ 1 และ 5 ที่ไม่พบว่ามีการผลิตสารที่มีฤทธิ์ยับยั้งเซลล์มะเร็งได้เลย จากการศึกษาผลของอาหารเลี้ยงเชื้อต่อการผลิตสารออกฤทธิ์ยับยั้งเซลล์มะเร็ง พบว่าอาหารชนิด CY ให้จำนวนสารสกัดหยาบที่มีฤทธิ์ยับยั้งเซลล์มะเร็งมากที่สุดคือ 23 ตัวอย่าง รองลงมาคืออาหารชนิด SK (11 ตัวอย่าง) RL (8 ตัวอย่าง) และ Vy/2 (7 ตัวอย่าง) ตามลำดับ และจากการศึกษาการแยก

สารที่มีฤทธิ์ยับยั้งเซลล์มะเร็งจากแบคทีเรียไกลดิงจำนวน 3 สายพันธุ์ ได้แก่ *Rapidithrix thailandica* (TISTR1768) *Rapidithrix thailandica* (TISTR1741) และ *Tenacibaculum mesophilum* (TISTR1782) พบว่าสามารถแยกสารชนิดต่างๆ ได้ทั้งหมด 9 สาร ประกอบด้วย cyclo(L-Pro-D-Phe) [**G1**], cyclo(L-Pro-L-Phe) [**G2**], *N*-(2-phenylethyl) acetamide [**G3**], cyclo(L-Pro-D-Tyr) [**G5**], cyclo(L-Pro-L-Tyr) [**G6**], cyclo(8-hydroxy-L-Pro-L-Leu) [**G7**], cyclo(8-hydroxy-L-Pro-D-Phe) [**G8**] และสารชนิดใหม่จำนวน 2 สารคือ 3-(2'-aminophenyl)-pyrrole [**G4**] และ 3-(2-Amino-phenyl)-5-methoxy-1,5-dihydro-pyrrol-2-one [**G9**] และจากการนำสารที่แยกได้จำนวน 4 สาร คือ cyclo(L-Pro-D-Phe) [**G1**], cyclo(L-Pro-L-Phe) [**G2**], *N*-(2-phenylethyl) acetamide [**G3**] และ 3-(2'-aminophenyl)-pyrrole [**G4**] มาทดสอบฤทธิ์ยับยั้งเซลล์มะเร็ง (IC_{50}) พบว่าสาร *N*-(2-phenylethyl) acetamide [**G3**] และ 3-(2'-aminophenyl)-pyrrole [**G4**] มีฤทธิ์ยับยั้งเซลล์มะเร็งในระดับปานกลาง ขณะที่สาร cyclo(L-Pro-D-Phe) [**G1**] และ cyclo(L-Pro-L-Phe) [**G2**] ไม่มีฤทธิ์ยับยั้งเซลล์มะเร็ง

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ABSTRACT

Twenty-seven strains of marine gliding bacteria were isolated from the specimens collected from coastlines in the Gulf of Thailand. It was found that biofilms provided the highest number of isolates followed by sponges, plant materials, algae and crab carcass. Subculturing and micromanipulation techniques were the most appropriate purification and isolation techniques for gliding bacteria in this study. Based on 16S rRNA gene sequence and phylogenetic analyse, the representative isolates were identified and classified to six different groups within the *Cytophaga-Flavobacterium-Bacteriodes* (CFB) group. Because of their similarities in 16S rRNA gene sequences, Groups 1, 4, 5 and 6 were proposed as novel genera including *Rapidithrix thailandica* gen. nov., sp. nov. (Gr. 4), *Aureispira marina* gen. nov., sp. nov. (Gr. 5) and *Aureispira maritima* sp. nov. (Gr. 6). Group 2 was identified as *Tenacibaculum mesophilum* whereas group 3 could potentially be a new species within *Fulvivirga kasyanovii*. During the course of cytotoxcity screening, forty-two marine gliding bacterial isolates were cultivated in four cultivation media (Vy/2, RL 1, CY and SK) and were screened for cytotoxicity against four human cell lines including cervical cancer (HeLa), colon cancer (HT-29), oral cancer (KB) and breast adenocarcinoma (MCF-7). The results showed that the members of groups 2 and 4 were capable of producing metabolites with cytotoxicities against all cell lines whereas the members of group 6 and the unidentified group could produce crude extracts with cytotoxicites against only HeLa and HT-29. Interestingly, groups 1, 3 and 5 could not produce any crude extracts with cytotoxicity. The CY medium was shown to be the most suitable medium for cytotoxic metabolites production yielding 23 cytotoxic extracts, followed by SK (11 extracts), RL 1 (8 extracts) and Vy/2 (7 extracts). Moreover, CY medium was the only medium which gave the active crude extract against MCF-7. Three extracts obtained from *Rapidithrix thailandica* (TISTR1768), *Rapidithrix*

thailandica (TISTR1741) and *Tenacibaculum mesophilum* (TISTR1782) were submitted to the bioassay guided fractionation in order to isolate and determine the compounds present in the extract. Nine metabolites including cyclo(L-Pro-D-Phe) [**G1**], cyclo(L-Pro-L-Phe) [**G2**], *N*-(2-phenylethyl) acetamide [**G3**], cyclo(L-Pro-D-Tyr) [**G5**], cyclo(L-Pro-L-Tyr) [**G6**], cyclo(8-hydroxy-L-Pro-L-Leu) [**G7**], cyclo(8-hydroxy-L-Pro-D-Phe) [**G8**] and two novel compounds, 3-(2'-aminophenyl)-pyrrole [**G4**] and 3-(2-Amino-phenyl)-5-methoxy-1,5-dihydro-pyrrol-2-one [**G9**], were isolated. Based on IC_{50} values *N*-(2-phenylethyl) acetamide [**G3**] and 3-(2'-aminophenyl)-pyrrole [**G4**] were considered moderately active against all four cell lines and three cell lines (HT-29, KB and MCF-7), respectively, whereas cyclo(L-Pro-D-Phe) [**G1**] and cyclo(L-Pro-L-Phe) [**G2**] exhibited no cytotoxicity.

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LIST OF ABBREVIATIONS AND SYMBOLS

δ	chemical shift (in ppm)
ε	molar extinction coefficient
λ	wavelength
ν	wave number
br	broad (for NMR signals)
CFB	*Cytophaga-Flavobacterium-Bacteroides*
d	doublet (for NMR)
DMSO	dimethylsulfoxide
EIMS	electron-impact mass spectroscopy
EMEM	Earle's salt minimum essential medium
HeLa	cervical cancer
HMBC	heteronuclear multiple-bond multiple-quantum coherence
HMQC	heteronuclear multiple-quantum coherence
HPLC	high performance liquid chromatography
HR-EIMS	high-resolution electron-impact mass spectroscopy
HT-29	colon cancer
IC_{50}	inhibitory concentration at 50% of tested subject
IR	infrared
J	coupling constant
KB	oral cancer
m	multiplet (for NMR)
MCF-7	breast adenocarcinoma
m/z	mass-over-charge ratio
MIC	minimum inhibitory concentration
MS	mass spectroscopy
NMR	nuclear magnetic resonance
nOe	nuclear Overhauser effect
s	singlet (for NMR)

LIST OF ABBREVIATIONS AND SYMBOLS (Cont.)

SEM	scanning electron microscope
SRB	sulphorhodamine B
t	triplet (for NMR)
UV	ultraviolet

CHAPTER 1

INTRODUCTION

1.1 General introduction

Cancer has become a serious problem in human health because of its higest number of death. So far, most of anticancer drugs were obtained and developed from terrestrial plants and microorganisms especially actinomycetes (Lam, 2006). However, the rate of discovery of new compounds from terrestrial microorganisms has been decreasing (Lam, 2006). Additionally, some anticancer drugs also have serious side effects and show low efficacy to cure cancers. Therefore, there is a critical need for novel cytotoxic compounds from other potential sources. It has been estimated that the biological diversity in the ocean, which cover more than 70% of the earth's surface, is higher than in the tropical rainforests (Haefner, 2003). Due to the physical environments of marine environment such as salinity, temperature and pressure, marine organisms have adapted the biological and chemical processes through evolution causing the production of different types of bioactive compounds (Lam, 2006). The ecological studies of marine natural products have revealed that many of these compounds contribute to the survival of the producers (Cooper, 2004). So far, several marine organisms were investigated in the drug discovery program by either pharmaceutical industries or public research institutes. Many cytotoxic agents have been obtained from many marine organisms such as salicylihalamide from sponges (Boyd *et al*., 2001); bryostatin-1 from bryozoans (Curiel *et al*., 2001); ecteinascidin-743 from tunicates (Izbicka *et al*., 1998); salinosporamide A (Feling *et al*., 2003) and resistoflavine (Gorajana *et al*., 2007) from marine bacteria. Regardless of the increasing numbers of bioactive compounds from marine microorganisms, the understanding about diversity of marine microorganisms, especially marine bacteria, is rather limited. Most of the study on diversity of marine bacteria as the producers of bioactive compounds has been focusing only on marine derived actinomycetes.

The members of marine gliding bacteria are unique Gram-negative bacteria with gliding motility commonly found in marine environments (Hosoya *et al*., 2007; Srisukchayakul *et al*., 2007; Hosoya *et al*., 2006; Iizuka *et al*., 2003a; Iizuka *et al*., 2003b; Barbeyron *et al*., 2001; Nakagawa *et al*., 2001; Johansen *et al*., 1999; Iizuka *et al*., 1998). However, the study on biodiversity and secondary metabolites of marine gliding bacteria is limited. Only a few metabolites, haliangicin (Fudou *et al*., 2001), neoverrucosanes (Spyere *et al*., 2003), miuraenamides A and B (Iizuka *et al*., 2006), and marinoquinoline A (Kanjana-Opas *et al*., 2006) have been reported from marine gliding bacteria so far, suggesting that they are still the untapped resources for natural product and drug discovery.

This study aims to isolate, identify, and classify marine gliding bacteria isolated in Thailand and their potential use for screening of cytotoxic compounds against human cell lines (HeLa, HT-29, KB and MCF-7).

1.2 Review of Literatures

1. 2.1 General introduction of gliding bacteria

Gliding bacteria are the group of unique Gram-negative bacteria with various vegetative shapes including rod, spiral and filamentous. The members of this group share common characteristics of cell motion by gliding or creeping along surface and behaving as social organisms which can be recognized by the emergence of a diffusive swarm colony (Gaspari *et al*., 2005; Rachid *et al*., 2005; Iizuka *et al*., 2003; Reichenbach, 1999; Iizuka *et al*., 1998; Reichenbach and Dworkin, 1991). Gliding bacteria can be divided into two major groups; fruiting body forming (myxobacteria) and non-fruiting body forming (*Cytophaga-Flavobacterium-Bacteriodes* [CFB] and non-fruiting myxobacteria) (Jiang *et al*., 2007).

1.2.1.1 Myxobacteria

The vegetative cells of myxobacteria are normally unicellular, rod-shaped with blunt or tapering ends (Fig. 1) with high G+C content (>50 %). Because of their gliding motility, the colonies develop as thin, film-like, spreading swarms (Fig. 2), particularly on media that are low in organic constituents (lean media).

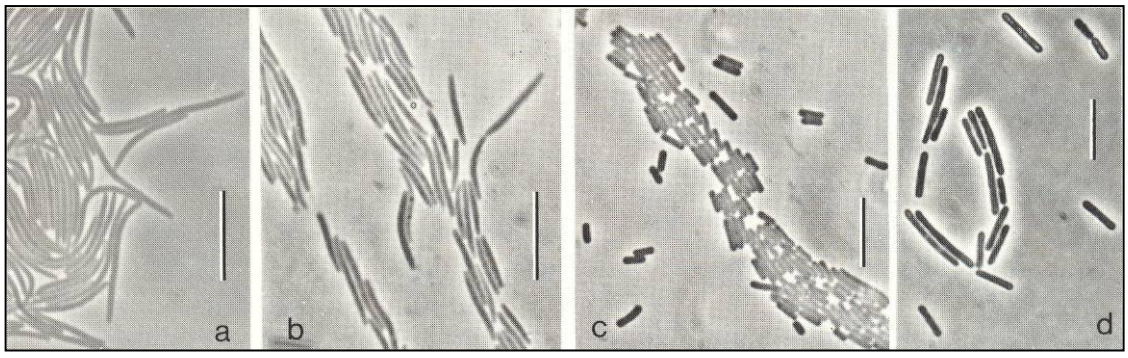


Figure 1. Various types of myxobacterial cells. (a and b) The tapering end type: (a) *Cystobacter ferrugineus*, cells from liquid culture. (b) *Stigmatella aurantiaca*, cells *in situ* on agar surface in chamber culture. (c and d) The blunt end type. (c) *Chondromyces crocatus* in chamber culture. (d) *Sorangium compositum* in chamber cuture. Bars = 10 μm.

Source: Reichenbach and Dworkin (1991)

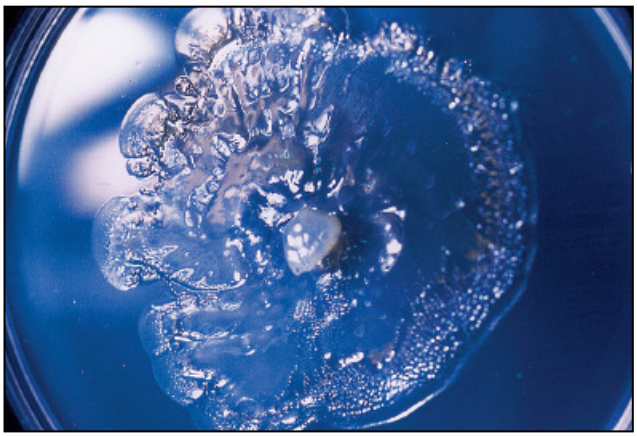


Figure 2. Swarm colony of myxobacteria, *Enhygromyxa salina*, which cultured for 10 days, 28 °C
Source: Iizuka *et al*. (2003b)

Under starving conditions, myxobacteria can undergo an impressive process of cooperative morphogenesis starting from the aggregation and piling up of vegetative cells resulting in a cell mass differentiation into fruiting bodies. During fruiting body maturation, a cellular differentiation takes place and vegetative cells convert into short, fat, optically refractile myxospores (Fig. 3). The formation of myxospores occurs inside the maturing sporangioles and fruiting bodies (Fig. 3 and 4). The vegetative rod cells undergo a cellular morphogenesis (Fig. 4) by becoming shorter and rounder shape and all vegetative cells turn into myxospores which are shorter and thicker than vegetative cells causing the resistance of harsh environments. These myxospores are structurally, physiologically and evolutionary different from other eubacterial endospores i.e. *Bacillus*'s endospores (Dawid, 2000). Endospore differentiation begins when a population of vegetative cells passes out of the exponential phase of growth, usually as a result of nutrient depletion. Typically one endospore is formed per vegetative cell. The mature spore is liberated by lysis of the mother cell (sporangium) in which it was formed. Endospores can be readily recognized microscopically by their intracellular site of formation and their extreme refractility.

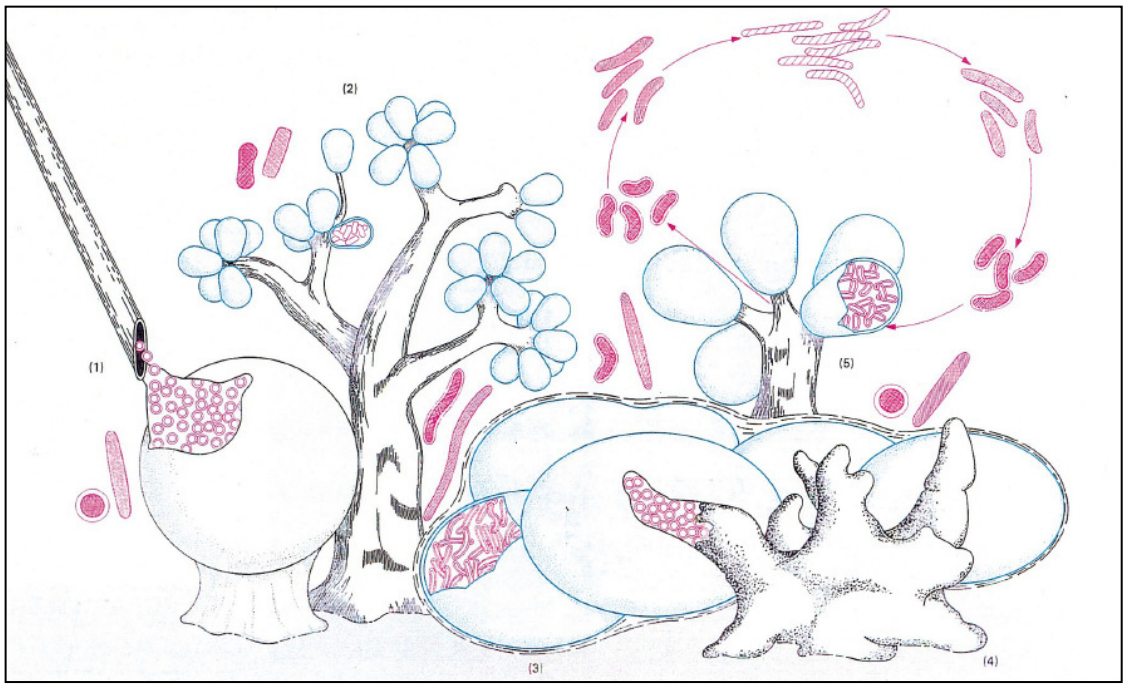


Figure 3. Fruiting bodies formation of myxobacteria, (1) *Myxococcus*: soft and slimy, so that material may be removed easily with the needle of a syringe. (2) *Chondromyces crocatus*. (3) *Cystobacter*. (4) *Corallococcus*: hard, cartilaginous slime. (5) *Stigmatella aurantiaca*: shown above the fruiting body in the cycle of cellular morphogenesis from myxospore to the vegetative cell and back to the myxospore

Source: Dawid (2000)

Myxobacterial fruiting bodies show various degrees of complexity, both morphologically and structurally. In their simplest appearances, e.g. in *Myxococcus*, the fruiting bodies are globular with soft mucous consistency (Fig. 4; Plate I, 1-4). Other genera (*Corallococcus*, *Archangium*) form rather bizarre shapes of hardened slime (Fig. 4; Plate I, 5, 6, 8). Most species enclose the myxospores in sporangioles that are surrounded by a defined wall. Sporangioles may occur singly or in groups. They are either embedded in the substrate (in case of *Angiococcus*, *Polyangium*, *Cystobacter*, *Sorangium*; Fig. 4, plate I, 7, 10, 16; Plate II, 17-19, 25, 29) or sit on hardened slime stems as one (in case of *Melittangium*, *Stigmatella*; Fig. 4, plate II, 23, 24, 26) or more sporangioles (in case of *Stigmatella*, *Chondromyces*; Fig. 4, plate II, 27, 28). The color of fruiting bodies is ranging from white, brown, bright yellow, orange, and red to lilac.

Fruiting body sizes also vary from 50 to 500 μm, allowing simple detection with naked eyes. However, interestingly it was demonstrated that myxospores, harvested from agar plate culture, could be kept on sterile filter paper strips in a dry condition for 10-25 years with good survival rate (Dawid, 2000). Other conditions that threaten the survival of vegetative cells, beside starvation of macronutrients, including temperature fluctuation, dessication, ultrasonification and ultraviolet radiation, can also stimulate myxospores production (Hartzell and White, 2001; Dawid, 2000). It was reported that nutrient starvation is the most common mechanism for inducing spore formation of myxobacteria (Hartzell and White, 2001). The common habitats of myxobacteria are slightly acidic to alkaline soil (pH 5-8) but sometimes they could also be found in acidic soil (pH 3.7). Frequently, myxobacteria could be found on the dung of herbivorous animals such as rabbit, decaying plant materials and on tree bark, and leaves (Dawid, 2000). Myxobacteria are generally considered as mesophilic bacteria with an optimal temperature at 30 °C. However, some psychrophilic myxobacteria were also reported from antarctic soil samples such as *Polyangium* and *Nannocystis,* which could only grow at the temperature range of 4-9 °C (Dawid, 2000).

Previously, myxobacteria were considered as typical soil bacteria (Reichenbach and Dworkin, 1991; Reichenbach, 1999). However, there have been an increasing number of reports on myxobacteria isolated from fresh and seawater, suggesting that myxobacteria are not restricted to terrestrial habitats. The examples of marine myxobacteria are *Nannocystis* spp., *Plesiocytis pacifica*, *Enhygromyxa salina*, *Myxococcus* sp., *Angiococcus* sp., *Corallococcus* sp. and *Cystobacter* sp. (Iizuka *et al*., 2003a; Iizuka *et al*., 2003b; Li *et al*., 2002; Reichenbach, 1999; Iizuka *et al*., 1998).

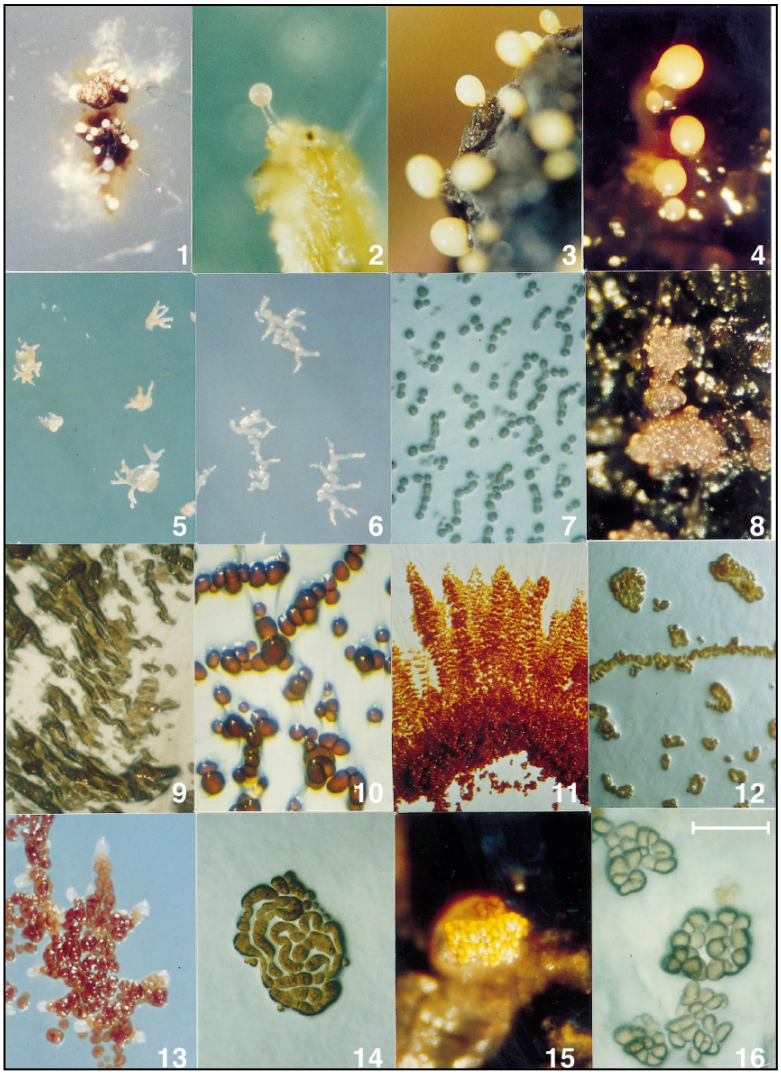


Figure 4. Plate I. Fruiting body varieties of myxobacteria. 1) *Myxococcus fulvus* on soil crumbs on coli-spot agar. 2) *Myxococcus stipitatus* on wood particles. 3) *Myxococcus virescens* on rabbit dung. 4) *Myxococcus xanthus* on peat. 5) *Corallococcus coralloides* on coli-spot agar. 6) *Corallococcus exiguous* on coli-spot agar. 7) *Angiococcus disciformis* on vy/2 agar. 8) *Archangium gephyra* on rabbit dung. 9) *Archangium serpens* on coli-spot agar. 10) *Cystobacter fuscus* on coli-spot agar. 11) *Cystobacter ferrugineus* on vy/2 agar. 12) *Cystobacter minus* on vy/2 agar. 13) *Cystobacter violaceus* on coli-spot agar. 14) *Cystobacter* sp. on coli-spot agar. 15) *Polyangium aureum* on rabbit dung. 16) *Polyangium compositum* on coli-spot agar. Bar = 240 µM.

Source: Dawid (2000)

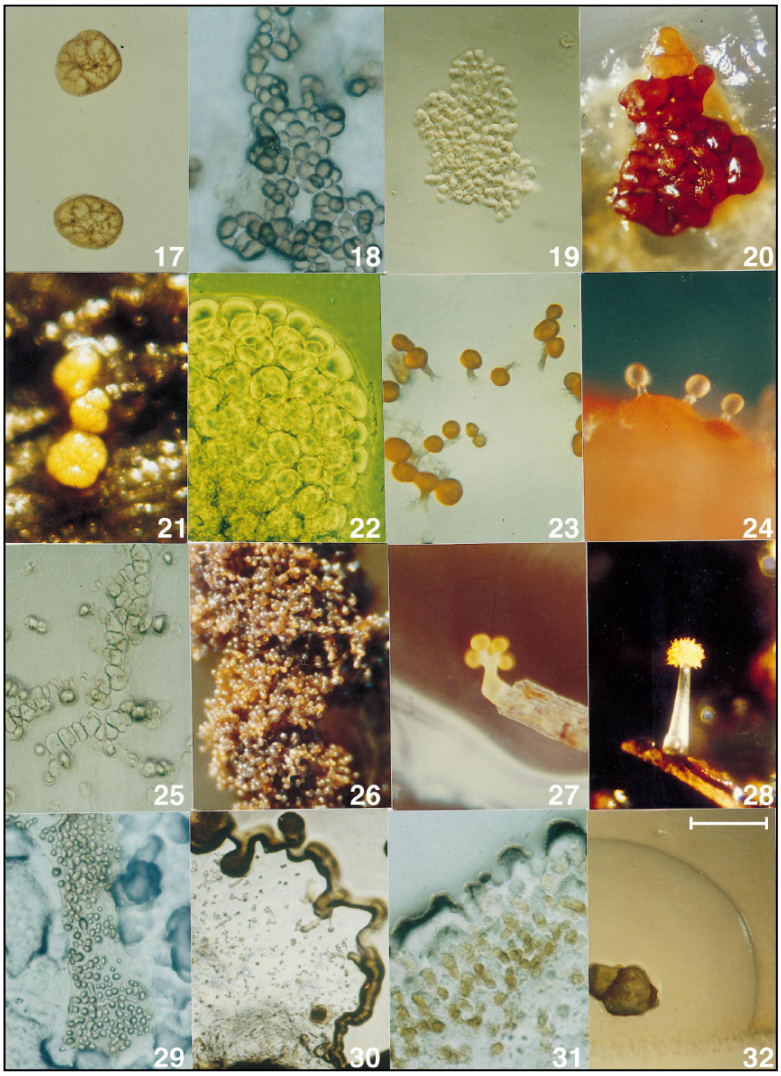


Figure 4. Plat II. Fruiting body varieties of myxobacteria (Cont.). 17) *Polyangium fumosum* on coli-spot agar. 18) *Polyangium sorediatum* on coli-spot agar. 19) *Polyangium spumosum* on vy/2 agar. 20) *Polyangium thaxteri* on rabbit dung. 21) *Polyangium* sp. on peat soil. 22) *Polyangium* sp. sporangioles. 23) *Melittangium boletus* on coli-spot agar. 24) *Melittangium lichenicola* on rabbit feces. 25) *Sorangium cellulosum* on STAN-6 agar. 26) *Stigmatella erecta* on coli-spot agar. 27) *Stigmatella aurantiaca* on wood particles. 28) *Chondromyces apiculatus* on rabbit dung. 29) *Nannocystis exedens* on coli-spot agar. 30) Psychrophilic myxobacteria, P type, swarm on coli-spot agar. 31) Psychrophilic myxobacteria, N type, swarm and sporangioles on coli-spot agar. 32) Psychrophilic myxobacteria, R type, on coli-spot agar. Bar = 300 µm.

Source: Dawid (2000)

1.2.1.2 *Cytophaga-Flavobacterium-Bacteriodes* (CFB)

Gliding bacteria in the group of CFB are all unicellular with low G+C content (<50 %). This morphology can be filamentous, spiral or rod-shaped with tapering or rounded ends (Fig. 5).

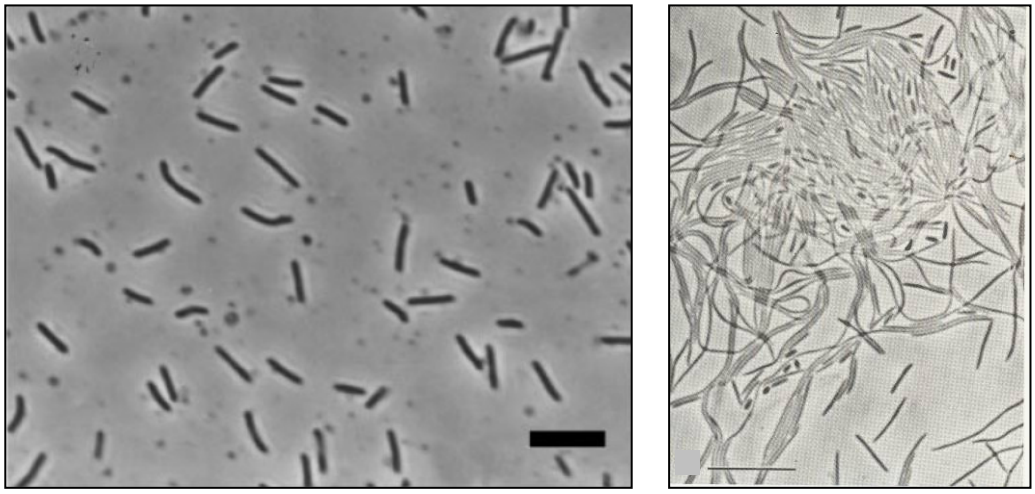


Figure 5. Vegetative cells of CFB. (Left) *Tenacibaculum amylolyticum*, cells grown on 1/5 LBM agar at 20 °C for 3 days. Bar = 5 μm. (Right) *Flexibacter filiformis*, cells grown on VY/2 agar. Bar = 10 μm.
Source: Reichenbach (1991); Suzuki *et al*. (2001)

While myxobacteria using fruiting body formation to survive the extreme conditions, CFB members do not possess this characteristic. The numbers of CFB can be found in terrestrial, aquatic and marine environments. They are also known as the decomposers of biomacromolecules such as cellulose, agar and chitin, suggesting that the members of CFB group could play an important role in carbon cycling in the environments. Reports on the member of CFB group isolated from marine habitats have been dramatically increasing during the past 20 years (Hosoya *et al*., 2007; Srisukchayakul *et al*., 2007; Hosoya *et al*., 2006; Nakagawa *et al*., 2001; Suzuki *et al*., 2001). Some of them were reported as fish or algal pathogens. These interactions between gliding bacteria and algae such as algicidal or agarolytic activities might lead to significant applications in marine biotecnology industry (Suzuki *et al*., 2001).

1.2.2. Isolation and classification of marine gliding bacteria

A few researchers have reported the isolation of gliding bacteria from marine environments (Iizuka *et al*., 2003; Lee *et al.*, 2003; Nedashkovskaya *et al.*, 2003; Li *et al.*, 2002; Barbeyron *et al.*, 2001; Suzuki *et al.,* 2001; Iizuka *et al*., 1998). This led to the begining of a new era for the studying on the biodiversities, secondary metabolites and applications of the untapped microbilogical resources.

Iizuka *et al*. (1998) were the first to report the presence of myxobacteria physiologically adapted to marine environments. They collected marine samples i.e. sand, wood specimens, animals (fishes, molluscs and crustaceans) and seaweeds from the intertidal areas of Hachijo-jima, Japan. The isolation method was based on a predatory activity using living *E. coli* smeared on an ASW-WCX agar plate which contained cyanocobalamin and cycloheximide. Within 2-4 weeks of incubation the swarm colonies of myxobacteria could be observed. The further purification, they cut the least contaminated area of the swarm colony into 0.5 × 0.5 mm pieces and transferred to a medium with fresh *E. coli* streaks. The procedure was repeated until the pure cultures could be obtained. The G+C mol percent, 16S rDNA sequence, phylogenetic analysis and the effect of NaCl on growth were used to identification and classification. In this study, the authors could obtained two strains of gliding bacteria including strains SHI-1 and SMP-2 which were characterized as rod-shaped with blunt ends whereas strain SMP-2 formed the fruiting body with chrome-yellow to lemon-yellow color. Within the fruiting body, spherical cells similar to the myxospores of *Nannocystis* stain were found. The strain SHI-1 showed spherical to oval cell clump with orange-white to light orange color. The major respiratory quinones of both isolates were identified as menaquinone 8 and the G+C contents of the DNA from strains SHI-1 and SMP-2 were 70.0 and 66.7 mol %, respectivety. The phylogenetic analysis demonstrated that the two marine gliding bacterial isolates were the members of the order *Myxococcales* and shared a common line of descent with the genus *Nannocystis*. The authors reported that the closest relatives for strain SHI-1 and SMP-2 were *N. exedens* and *N. exedens* with 89.3 and 82.9 % similarity, respectively (Iizuka *et al*., 1998). Interestingly, no previously reported myxobacterium was able to grow at the salinity of natural seawater. Therefore, this was the first myxobacteria isolated from marine habitat.

Shortly after that, marine gliding bacteria isolated from three Danish marine environments, including, Hirsholm, Ellekide Hage and Svaneka were described by Johansen and colleagues (1999). The isolates were obtainted from the surface of the marine benthic macroalga *Fucus serratus*, collected from the upper part of the sublittoral zone (1 m depth) and the surrounding seawater collected in the deeper part of the sublittoral zone (3-5 m depth). The algal specimens were homogenized with an Ultra-Turrax in sterile deionized water containing sea salts. The homogenates and the seawater samples were diluted and plated on CYT agar (1.0 g casein, 0.5 g yeast extract, 0.5 g $CaCl_2.H_2O$, 0.5 g $MgSO_4.H_2O$, 15.0 g agar, 1000 ml deionized water, pH 7.3). The single colonies were subcultured several times until the pure cultures were obtained. Eventually, nine strains of CFB were obtained from the surface of *Fucus serrstus* and two strains were obtained from the seawater samples. The authors reported that the colony morphology of the two new species varied significantly depending on the cultivating temperature and substrates. All strains grew on TSA (tryptic soy agar) supplemented with 10-50% sea salts but a few growth was observed at 2.5% sea salts and no growth was observed without the addition of sea salts. Optimal pH was between 7 and 8 and no growth was observed at pH below 6. Only little growth was found at pH 9 after 10 days of incubation. The isolates were identified as Gram negative, psychro and halophilic bacteria with flexirubin-negative, aerobic, catalase-positive and oxidase-negative characteristics. The investigators described that all strains could utilize D-fructose, L-fructose and α-ketobutyric acid and degrade alginic acid, carrageenan, starch and autoclaved yeast cells. Phylogenetic analysis of 16S rRNA sequences of two isolated strains including strain NN015840^T and NN015860^T and *Cytophaga lytica* showed the association at the genus level. However, the authors reported that their DNA-DNA hybridization revealed that the new strain and *Cytophaga lytica* ATCC 23178^T were clearly distinct from each other. The authors suggested that these two strains should be reassigned to the new genus *Cellulophaga* gen. nov. comprising two new species *Cellulophaga baltica* gen. nov., sp. nov. (NN015840^T = LMG 18535^T) and *Cellulophaga fucicola* gen. nov., sp. nov. (NN015860^T = LMG 18536^T) (Johansen *et al.*, 1999).

Later, Barbeyron and co-workers (2001) reported the isolation of novel marine gliding bacterium, *Zobellia galactanovorans* gen. nov., sp. nov., from the red alga (*Delesseria sanguinea*) collected in the English Channel near Roscoff (Brittany, France). This novel gliding bacterium was a mesophilic, aerobic, non-flagellated, long rod shape, which formed yellow colonies and could degrade both agar and carrageenan. The G+C content of its genomic DNA was 42-43 mol%. Phylogenetic analysis of 16S rRNA gene sequences, using 8F and 1492R primers, indicated that this isolate was closely related to *Cytophaga uliginosa* DSM 2061 with a sequence identity of 99.5%. However, the authors described that the total protein profile of the newly isolated strain was different from *Cytophaga uliginosa* and DNA-DNA hybridization experiment confirmed that the new strain constituted a new distinct species. Based on its phenotypic features and the phylogenetic relationship, the authors proposed a new genus that designated as *Zobellia* to include *Zobellia alactanovorans* and *Cytophaga uliginosa*, which was later renamed to *Zobellia uliginosa* (Barbeyron *et al.*, 2001).

Another study on novel the marine *Cytophaga*-like bacteria isolated from sponge and green algae collected from the coast of Japan and Palau was reported by Suzuki *et al*. (2001). They described the phylogenetic relationship of the isolates by using *gyrB* gene and translated peptide sequences (GyrB) in addition to 16S rDNA sequences. The authors suggested that 16S rRNA sequences might be a powerful tool for bacterial identification but the resolution of 16S rRNA sequence analysis might not be sufficient to distinguish the closely related organisms. Whereas the protein-encoding genes, which evolve faster than rRNA gene (rDNA), were could potentially be used for a phylogenetic analysis with a higher resolution (Yamamoto and Harayama, 1996 referred by Suzuki *et al*., 2001). Yamamoto and Harayama (1995) had shown that a phylogenetic analysis based on the *gyr*B sequence of *Pseudomonas putida* had a greater degree of resolution than that of 16S rRNA sequence. Suzuki and colleagues (2001) reported gliding bacterial strains isolated from sponge and green algae which were closely related to the previously characterized marine *Flexibacter* species, *F. maritimus* and *F. ovolyticus.* These *Flexibacter* species are distantly related to *F. flexilis*, the type species of the genus *Flexibacter*, and phylogenetically belong to the family *Flavobacteriaceae* (based on analysis using both 16S rDNA and GyrB sequences). The authors suggested that the isolates possessed MK-6 as the respiratory quinone system and contained zeaxanthin as the major carotenoid pigment, contained

β-cryptoxanthin and *β*-carotene as minor components. The isolates produced bright yellow colonies on 1/5 LBM agar and did not adhere to the agar plate. After 5 days incubation, the sizes of the colonies were between 23 and 60 mm in diameter. They grew at temperatures between 20 and 35 °C, the optimum temperature being between 27 and 33 °C. The strains grew well on the 1/5 LBM medium containing 70% (v/v) sea water. These bacteria could utilize certain amino acids as sole carbon and nitrogen sources, and require an organic nitrogen source for growth. According to the phylogenetic, chemotaxonomic and phenotypic characteristics of the two species, the authors suggested that they should be transferred to the new genus *Tenacibaculum* including two new species *T. maritimum* and *T. ovolyticum*. Later on, two additional new species of this genus *T. mesophilum* gen. nov., sp. nov. and *T. amylolyticum* gen. nov., sp. nov., isolated from sponges and macro-algae, respectively were also reported by the same group (Suzuki *et al*. 2001). They also suggested that the resolution of *gyrB* sequences was superior to that of 16S rDNA sequences and the grouping based on the *gyrB* phylogram was consistent with DNA-DNA hybridization results (Suzuki *et al*., 2001).

Nakagawa *et al.* (2001) reported the phylogenetic diversity of *Cytophaga*-like strains isolated from Amami-O-Shima (Amami Islet) and Iriomote Shima (Iriomote Islet) in the sub-tropical zone of Japan. The almost complete 16S rRNA gene (16S rDNA) was amplified by PCR with the primers 9F and 1541R. The partial 16S rDNA sequences (500 bps) were analysed with the oligonucleotide primers 9F and 536R for initial search of the taxonomic position of the isolates. In addition to 9F and 536R, the oligonucleotide primers used to sequence the full length of amplified 16S rDNA were 339F, 785F, 1099F, 802R, 1115R and 1541R. The initial phylogenetic analyses to search for the taxonomic positions of all isolates were examined with the partial sequences of 16S rDNA (500 bps). The authors reported that thirty-nine strains belonged to the *Cytophaga-Flavobacteria-Bacteroides* (CFB) phylum and could be divided into 15 groups (cfb 1 to 15) and 15 sub-groups. The investigators described that fourteen groups were located within the family *Flavobacteriaceae* and were affiliated with various taxa i.e. cfb 2 clustered with the genus *Salegentibacter* and *Cytophaga latercula*; cfb 4 clustered with *Psychroserpens*; cfb 6, 7, and cfb 8 clustered with *Cellulophaga*; cfb 9 clustered with *Cytophaga marinoflava*; cfb 11 clustered with *Polaribacter*; cfb 12 clustered with the new genus *Tenacibaculum*; cfb 13, 14, and 15 clustered with *Flavobacterium*; cfb 3 formed a sister group of *Psychroflexus*; cfb 5 formed a sister

group of *Capnocytophaga* and *Coenonia*; and cfb 10 formed a sister group of *Cellulophaga* and *Cytophaga marinoflava.* The remaining group, cfb 1, was not included in the family *Flavobacteriaceae* and constituted a distinct phylogenetic lineage as a sister group of *Cyclobacterium marinum*. Only cfb 8 exhibited an exact match with 16S rRNA sequences deposited in the GenBank/EMBL/DDBJ databases. The authors also suggested that almost all groups were considered to represent new genera and/or species. From phylogenetic results, they described that the biodiversity of the CFB phylum is much wider than what was previously thought (Nakagawa *et al.*, 2001).

Phylogenetic structure of the genera *Flexibacter, Flexithrix,* and *Microscilla* deduced from 16S rRNA sequence analysis was reported by Nakagawa and co-workers (2002). The almost complete 16S rRNA gene (16S rDNA) was amplified by PCR with the same primers as described in Nakagawa and colleagues (2001) as well as the set of oligonucleotide primers used to sequence the full length of amplified 16S rDNA. The results of 16S rRNA sequence analysis demonstrated the extreme heterogeneity of the genera *Flexibacter* and *Microscilla.* The authors proposed that the isolates were diverged into 24 distinct lines of descent that were remote from each other at the genus level or higher. *Flexibacter* strains were scattered across the *Cytophaga-Flavobacteria-Bacteroides* phylum and divided into 20 phylogenetic groups. Genus *Microscilla* was separated into 5 groups. Nakagawa and co-workers further suggested that each genus should be restricted to only the type species. *Flexithrix dorotheae*, the type species of the genus *Flexithrix*, clustered with *Flexibacter aggregans.* The heterogeneity was found not only within genera but also within species. *Flexibacter aggregans*, *Flexibacter aurantiacus*, *Flexibacter flexilis*, *Flexibacter roseolus*, *Flexibacter tractuosus*, and "*Microscilla sericea*" each contained phylogenetically distant strains. The authors therefore suggested that the taxonomic concept of the genera *Flexibacter*, *Flexithrix*, and *Microscilla* should be reorganized to *Flexibacter*. However, the investigators reported the comprehensive taxonomic studies on these genera have not yet been performed because only a few phenotypic, physiologic, and chemotaxonomic features have been reported so far in the future. Accumulation of these data will definitely lead to the improved taxonomy and classification of these organisms (Nakagawa *et al*., 2002).

Kurahashi and Yokota (2002) proposed that bacteria associated with marine organisms, especially molluscs, could be important sources for novel bacteria. During the expedition to collect marine organisms from the coasts of the Kanto area in Japan, they were able to isolate a total of 116 bacterial isolates of bacteria from the intestines of 19 species of marine organisms includings molluscs, fishes and protochordata. Partial sequencing of 16S rDNA revealed that most of the isolates belonged to the gamma subclass of the *Proteobacteria* and *Cytophaga-Flavobacterium-Bacteroides* groups. Further description revealed that 5 strains belonged to the *Cytophaga-Flavobacterium-Bacteroides* group with less than 94% similarity. Phylogenetic positions of the strains were also independent from the neighboring taxa, suggesting the formation of a novel lineage. This result clearly indicated that marine organisms could be the potential sources of novel groups of bacteria.

Li *et al*. (2002) reported the isolation of salt-tolerant myxobacteria from marine samples i.e. seawater, marine sands, and sediments from Shandong province, China. Sand and sediment samples were air dried immediately after collecting and the isolation was performed based on the predatory activity of gliding bacteria (Reichenbach and Dworkin, 1991). Living *E. coli* was smeared on modified WAT medium agar plate ($CaCl_2.2H_2O$ 0.05%, Agar 1.5% in seawater) and approximately 0.2 g of air dried sand or sediment samples were directly spreaded on modified WAT medium agar plates and incubated at 30 °C until the myxobacterial fruiting body structures could be detected by a dissecting and a phase contrast microscopes. The swarm colonies were carefully scraped to glass slides for an examination. Salt requirement of marine myxobacteria were tested by spreading the isolates on media consisting entirely of seawater (3.4% salt concentration) and observing the appearance of myxobacterial fruiting bodies. The investigators reported that no colony of myxobacteria was found when prepared the isolation medium with diluted seawater (<60% seawater or lower than 2.0% salts concentration) and some myxobacterial fruiting bodies could actually be formed. The authors reported that salinity could play an important role on the growth and fruiting body formation of marine myxobacteria. Low salt condition might be unsuitable for growth that leads to stimulation of fruiting body formation in order to prevent the community from the unsuitable environment. According to their identification, these isolates belonged to the genera *Myxococcus, Angiococcus, Corallococcus* and *Cystobacter*. The authors described that the normal isolation techniques using full strength

seawater could lead to the problem of finding fruiting body formation and misidentification of the isolate (Li *et al*., 2002).

Zhang and co-workers (2003) compared the isolation and purification methods for terrestrial myxobacteria which may be adaptable able for the isolation of marine myxobacteria. These methods included the selective isolation with crystal violet medium, baiting technique, transferring of cover slip, transferring of fruiting body and re-inoculation of sterile rabbit dung pellets. The results are shown in Table 1.

Table 1. Number of myxobacterial isolates obtained from each isolation technique

Method of purification	Number of isolate	Number of pure culture	Proportion of pure culture (%)	Period of purification (month)
Transferring of fruiting body	115	3	2.6	1 - 2
Freezing and heating	69	1	1.5	1 - 2
Antibiotic	42	1	2.4	1 - 2
Freezing, heating and multiple treatment with antibiotics	19	1	5.3	1 - 2
Streak culture	83	-	-	-
Transferring of cover slip	57	-	-	-
Inoculation with sediment growth	8	4	50.0	1
Selective isolation with crystal violet medium	85	36	42.5	1
Second baiting technique	27	23	69.7	0.5
Micromanipulator	16	1	6.3	0.5

Source: Zhang e*t al*. 2003

Zhang and co-workers described that one of interesting techniques was to introduce sterile rabbit dung as a natural growth stimulating substance in an isolation medium to induce the fruiting body formation that facilitates the purification of the isolate. It was found that streak culture technique and transferring of cover slip could not yield the pure culture of myxobacteria in this study whereas using crystal violet medium and micromanipulator yielded 42.5% and 6.3% propotion of pure culture, respectively (Zhang *et al*., 2003).

Novel marine gliding bacterium, *Reichenbachia agariperforans* gen. nov., sp. nov., was isolated from seawater sample in the Amursky Bay of the Gulf of Peter the Great, Sea of Japan by Nedashkovskaya and co-workers (2003). The 16S rDNA sequence analysis indicated that this novel strain was a member of the phylum *Cytophaga-Flavobacterium-Bacteroides* with rod-shaped cells, gliding motility, and non-diffusible orange pigment. There was no endospore or resting stages but it required Na^{+} ions and oxygen for growth. The G+C content was 44.5 mol% and the major respiratory quinone was MK-7. Their main cellular fatty acids were straight-chain unsaturated and branched-chain saturated fatty acids, $C_{15:0}$ and $C_{16:1(n-7)}$ (Nedashkovskaya *et al*., 2003).

Iizuka and co-workers (2003a) reported two strains of a novel myxobacteria (SIR-1 and SHI-1) isolated from the Japanese coast located in the Pacific subtropical zone. The strains were chemoheterotrophic and strictly aerobic with common characteristics associated with myxobacteria, such as gliding motility, bacteriolytic activity and fruiting body formation (Fig. 6). Both strains required NaCl for growth with an optimum concentration of 2.0-3.0% (w/v), which were comparable to that of seawater. These isolates demonstrated the presence of alkaline phosphatase, acid phosphatase and naphthol-AS-BI phosphohydrolase and growth occurred at temperatures between 15 and 32 °C, with an optimum around 28-30 °C. The optimum pH range for growth was 7.5-8.5. In addition, these strains also required other cationic components of seawater such as Mg^{2+}, Ca^{2+} and K^{+} and the major respiratory quinone was MK-8. The authors reported that cellular fatty acid profile was characterized by the predominance of iso-$C_{15:0}$ with anteiso-$C_{16:0}$, anteiso-$C_{17:0}$, and a long-chain polyunsaturated fatty acid ($C_{20:4}$). The G+C content of the genomic DNA of strains SIR-1 and SHI-1 were between 69.3 and 70.0 mol%. The sequence similarity between the two strains was 99.5%, suggesting they should be classified as a single species or as very closely related species. These two strains shared almost identical 16S rDNA

sequences and clustered with the genus *Nannocystis*. However, the authors described that phylogenetic distance between the novel strains and the genus *Nannocystis* was large enough to warrant their different generic allocation. Thus, the investigators proposed a novel genus and species *Plesiocytis pacifica* gen. nov., sp. nov. (Iizuka *et al.*, 2003a).

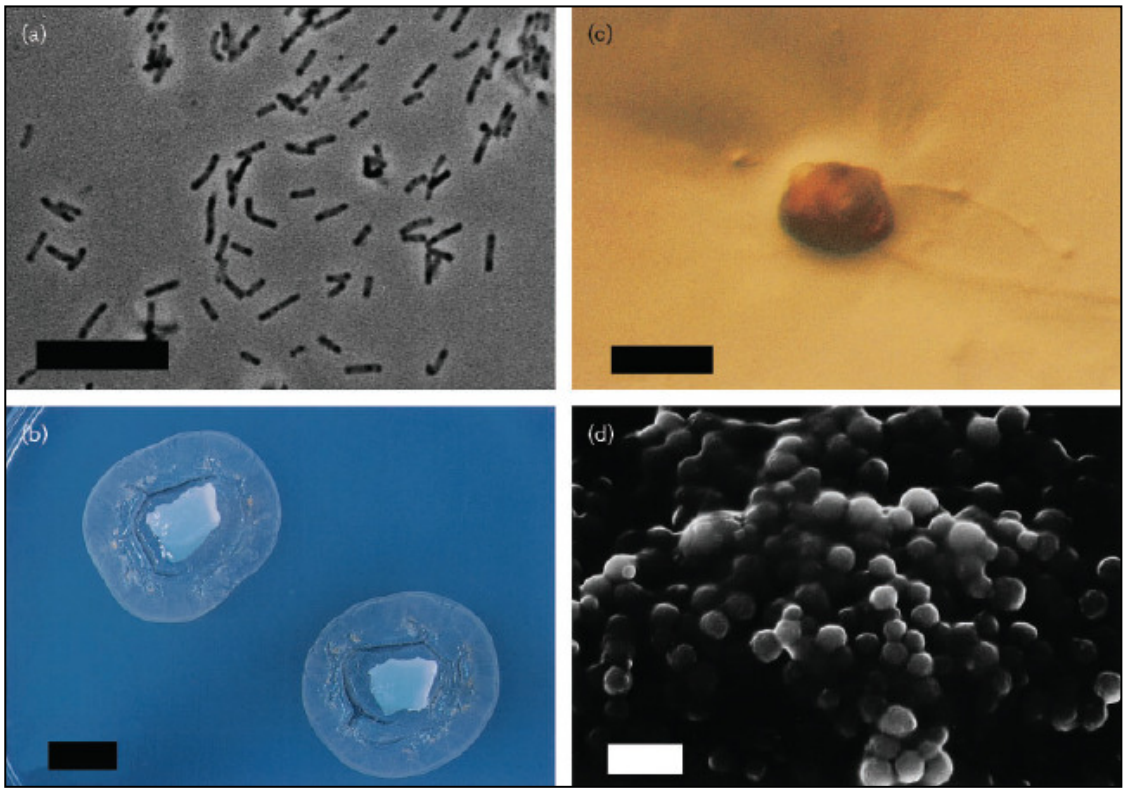


Figure 6. The morphological photographs of *Plesiocystis pacifica* (strain SIR-1). (a) Phase-contrast micrograph of vegetative cells cultured in casein/SWS liquid medium for 3 days at 28 °C, observed under oil immersion. Bar = 10 µm. (b) Swarming colonies formed around inoculated agar blocks on 1/3 CY/SWS agar cultivated for 5 days at 28 °C. The agar gel within the swarms was cleaved but not liquefied. Bar = 1.0 cm. (c) Solitary fruiting body on the surface of Vy2/SWS agar after 10-14 days cultivation at 28°C, observed under a dissecting microscope. Bar = 100 µm. (d) Scanning electron micrograph of the spherical myxospores. A fruiting body on Vy2/SWS agar was removed onto a small filter-paper strip and crushed with a needle tip. Bar = 1.0 µm

Source: Iizuka *et al*. (2003a)

Iizuka and colleagues (2003b) reported the isolation of a novel slightly halophilic myxobacterium, *Enhygromyxa salina* gen. nov., sp. nov. from the mud, sand and algal samples obtained from the coastal area of Japan. All of the isolates were aerobic, Gram-negative, rod-shaped with gliding motility, bacteriolytic activity, and fruiting body formation. The isolates also required NaCl at the concentrations of 1.0-2.0% (w/v), and other divalent cationic components of seawater such as Mg^{2+} or Ca^{2+} for growth. The major respiratory quinone was found to be MK-7. The G+C content of genomic DNA ranged from 65.6 to 67.4 mol%. The isolates shared almost identical 16S rDNA sequences, and clustered with a recently described marine myxobacterium *Plesiocystis pacifica* as their closest relative on a phylogenetic tree (95.9-96.0% similarity). The authors described physiological and chemotaxonomic differences between the new strains and strains of the genus *Plesiocystis* justify the proposal of a new genus, *Enhygromyxa salina* gen. nov., sp. nov. Their mophology, swarm colony and fruiting body are shown in Figure 7 (Iizuka *et al*., 2003b).

A new species of the CFB group, $LA1^T$, isolated from the Hawaiian hypersaline lake was reported by Donachie *et al*. (2004). Cell morphology was described as fine rods to short filaments. The isolate could grow in 50% strength marine broth containing 7.5-10% (w/v) NaCl. The major fatty acids of $LA1^T$ grown at 15 and 30 °C were 12-methyl tetradecanoic acid and 13-methyl tetradecanoic acid, respectively. The closest described neighbour, based on 16S rRNA gene sequence, was *Psychroflexus torquis* ACAM 623^T (94.4% over 1423 bases), an obligate psychrophile from Antarctic sea-ice. Phenotypic and genotypic analyses, including DNA hybridization (revealed only 4% DNA reassociation between $LA1^T$ and *P. torquis*), indicated that $LA1^T$ could be assigned to the genus *Psychroflexus*. However, based on significant differences, including growth at 43 °C, it should be assigned to a novel species, *Psychroflexus tropicus* sp. nov. (Donachie *et al*., 2004).

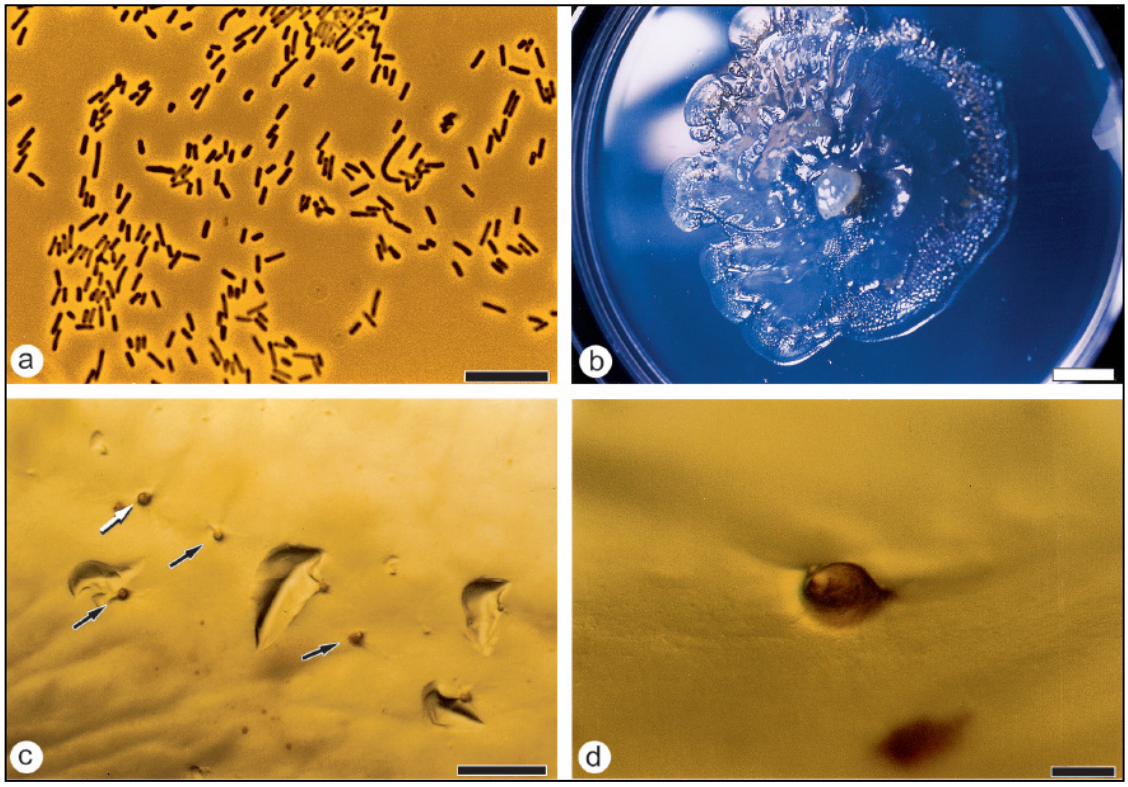


Figure 7. The morphological photographs of *Enhygromyxa salina* (strain SHK-1^T). a: Phase-contrast micrograph of vegetative cells cultured in casein/SWS liquid medium for 5 days at 28 °C. Bar =10 µm. b: Swarming colony formed around inoculated agar block on 1/3CY/SWS agar for 10 days at 28 °C. Bar = 1.0 cm. c: Solitary fruiting bodies (indicated by arrows), on the surface of Vy2/SWS agar after 10 days cultivation at 28 °C, observed under dissecting microscope. Agar surface near the fruiting bodies were sometimes cleaved. Bar = 1.0 mm d: Side view of a fruiting body observed under dissecting microscope. Bar = 100 µm

Source: Iizuka *et al*. (2003b)

A novel *Tenacibaculum*-like bacterial strain, SMK-4^T, isolated from tidal flat sediment in Korea, was reported by Jung and co-workers (2006). Strain SMK-4^T was characterized as rod-shaped, Gram-negative with pale yellow-pigmented. Its optimum temperature was 30-37 °C in the presence of 2-3 % (w/v) NaCl. MK-6 was the predominant menaquinone and iso-$C_{15:0}$, iso-$C_{16:0}$ 3-OH and $C_{16:1}\omega 7c$ and/or iso-$C_{15:0}$ 2-OH were major fatty acids (>10 % of total fatty acids) in the cells. The G+C content was 33.6 mol% and the phylogenetic tree based on 16S rRNA gene sequences indicated that strain SMK-4^T fell within the evolutionary radiation

encompassed by the genus *Tenacibaculum*. Strain SMK-4^T exhibited 16S rRNA gene sequence similarity level of 95.2-98.6 % with respect to the type strains of recognized *Tenacibaculum* species. DNA-DNA relatedness levels and differential phenotypic properties made it possible to categorize strain SMK-4^T as a species that is separate from previously described *Tenacibaculum* species. On the basis of phenotypic properties and phylogenetic and genetic distinctiveness, strain SMK-4^T was proposed as *Tenacibaculum aestuarii* sp. nov. (Jung *et al*., 2006).

Choi and colleagues (2006) reported a new species of gliding bacterium of the genus *Tenacibaculum.* A rod-shaped bacterium with pale yellow colony, designated as CL-TF13^T was isolated from a tidal flat in Ganghwa, Korea. The analysis of the 16S rRNA gene sequence revealed an affiliation with the genus *Tenacibaculum* with similarities between 94.2 to 97.4 % and the novel species was proposed as *T. litoreum* sp. nov. The strain was able to grow in NaCl and temperature range of 3-5 % and 5-40 °C, respectively, while the optimum pH were between 6 and 10. The major fatty acids were $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH (19·6 %), iso-$C_{15:0}$ (18·8 %) and iso-$C_{17:0}$ 3-OH (13.6 %). Fatty acids such as $C_{18:3}\omega6c$ (6, 9, 12) (1.5 %) and summed feature 4 (iso I- and/or anteiso B-$C_{17:1}$, 1.3 %) were uniquely found in minor quantities in CL-TF13^T similar to other *Tenacibaculum* species (Choi *et al*., 2006).

Hosoya and colleagues (2006) reported three novel strains of gliding bacteria, TISTR1719^T, TISTR1728 and TISTR1731 isolated from a marine sponge and algae from the southern coastline of Thailand. A phylogenetic analysis, based on 16S rRNA gene sequences, formed a distinct lineage within the family '*Saprospiraceae*' of the phylum *Bacteroidetes* and were somewhat related to members of the genus *Saprospira*. The G+C contents of the isolates were in the range of 38-39 mol%. The major respiratory quinone was MK-7. It was interesting to note that the predominant cellular fatty acids were 20:4$\omega6c$ (arachidonic acid), 16:0 and iso-17:0 which is uncommon among bacteria. On the basis of morphological, physiological and chemotaxonomic characteristics together with DNA-DNA hybridization data and 16S rRNA gene sequences, the authors proposed the novel genus and species as *Aureispira marina* gen. nov., sp. nov. because of its spiral shape and yellow pigment (Hosoya *et al*., 2006).

Recently, Sheu and co-workers (2007) had isolated a yellow-colour, aerobic, Gram-negative, rod-shaped bacterial strain, designated as B-I^T, from the water of a shrimp (*Litopenaeus vannamei*) mariculture pond in Taiwan. The highest sequence similarity of strain B-I^T (93.2-96.1 %) was to the members of the genus *Tenacibaculum*. The novel species was proposed as *T. litopenaei* sp. nov. The major fatty acids were iso-$C_{15:0}$ (22 %), $C_{16:1}\omega7c$ and/or iso-$C_{15:0}$ 2-OH (21.3 %), iso-$C_{17:0}$ 3-OH (12.7 %) and iso-$C_{15:1}$ (8.7 %). The G+C content of the genomic DNA was 35.2 mol% (Sheu *et al*., 2007).

Hosoya and co-workers (2007) also reported another novel gliding marine bacterium (TISTR1726^T) that isolated from marine barnacle debris from the southern coastline of Thailand. A phylogenetic analysis based on 16S rRNA gene sequences showed that the isolate formed a distinct lineage within the recently proposed genus *Aureispira*. Base on DNA-DNA hybridization, physiological and chemotaxonomic analyses and 16S rRNA gene sequence comparison, TISTR1726^T was proposed as *Aureispira maritima* sp. nov. (Hosoya *et al*., 2007).

Another report of the discovery of marine gliding bacteria in Thailand came from Srisukchayakul *et al*. (2007). They reported the discovery of a novel marine gliding bacterium, *Rapidithrix thailandica* gen. nov., sp. nov., isolated from the southern coastline of Thailand. The taxonomic positions of three isolates of marine gliding bacteria, TISTR 1736, TISTR 1741 and TISTR 1750^T, were evaluated by using a polyphasic approach. Phylogenetic analysis based on 16S rRNA gene sequences indicated that the three isolates formed a distinct lineage within the family *Flammeovirgaceae*, phylum *Bacteroidetes*, and were related to the genus *Flexithrix*. The G+C content of the isolates were in the range of 40-43 mol%. The major respiratory quinone was MK-7 which the major cellular fatty acids were 16:1$\omega5c$ (*cis*-5-hexadecenoic acid) and 15:0 (pentadecanoic acid) with major hydroxyl fatty acids as 3-OH 17:0 (3-hydroxyheptadecanoic acid), 3-OH 15:0 (3-hydroxypentadecanoic acid) and 3-OH 16:0 (3-hydroxyhexadecanoic acid). On the basis of phenotypic, chemotaxonomic, genotypic and phylogenetic data, they were proposed as *Rapidithrix thailandica* gen. nov., sp. Nov

Nedashkovskaya *et al*. (2007) reported a novel gliding bacterium isolated from seawater in a mussel farm with the G+C content of 59.9 mol%. Phylogenetic analysis based on 16S rRNA gene sequences indicated that this strain formed a cluster with the misclassified strains *Flexibacter aggregans* NBRC 15974, *Flexibacter tractuosus* NBRC 16035 and with the type strains of *Reichenbachiella agariperforans* and *Roseivirga ehrenbergii* with levels of similarity of 95.9, 94.4, 92.0 and 91.8 %, respectively. On the basis of its phenotypic, chemotaxonomic, genotypic and phylogenetic characteristics, a novel species of a new genus in the phylum *Bacteroidetes* for which the name *Fulvivirga kasyanovii* gen. nov., sp. nov. was proposed (Nedashkovskaya *et al*., 2007).

The latest report of a novel marine gliding bacteria came from Asker *et al*. (2007), of which *Mesoflavibacter zeaxanthinifaciens* gen. nov., sp. nov. was isolated from the seawater near Kamakura city, Japan. Its unique characteristic was the major carotenoid pigment as zeaxanthin instead of flexirubin-type pigments.

1.2.3 Bioactive compounds produced by myxobacteria

Myxobacteria are known to prey upon other microorganisms by lysis which provides nutrients from the lysed cells to the predator (Iizuka *et al.*, 1998). This may be one of the reasons why myxobacteria produce the varieties of bioactive compounds. In the past decade, more than 40 basic structures and 200 structural variants had been isolated from myxobacteria, most of which were fully characterized as bioactive compounds (Reichenbach and Dworkin, 1991).

It is interesting to note that a few compounds produced by myxobacteria were contained with chlorine and nitro groups. Tartrolon produced by *Sorangium cellulosum*, was one of only four natural products known to contain boron (Irschik *et al*., 1995). More interestingly, *Sorangium cellulosum* alone had produced more than 30 new compounds, only a few of which had been discovered in other myxobacteria. Similarly, *Chondromyces crocatus* produced at least six to seven different compounds whereas *Stigmatella aurantiaca* produced stigmatellin, myxalamid and at least four different aurachins that showed potent antimicrobial activities. It was found that one particular strain of myxobacterium might produce different compounds in varying combinations, e.g., one strain of *Sorangium cellulosum* could produce sorangicin, disorazol, chivosazol and

sulfangolid whereas another produced disorazol, icumazol and soraphen (Reichenbach, 2001). It can be concluded that myxobacteria are among one of the most prolific group of bacteria in terms of secondary metabolite production.

Reichenbach (2001) reported that among 80 different basic structures and 450 structural variants produced by myxobacteria, were either moderately lipophilic, linear or cyclic polyketides or peptides. Only one modified sugar and one modified nucleotide substances have been reported from myxobacteria so far. Polyketides obtained from myxobacteria belong to uniquely different classes i.e. macrocyclic lactones, lactams, macrolides with a sugar attached, polyether, polyenic compounds, alkaloids, and aromatic compounds. The peptides often found were depsipeptides containing hydroxy acids in addition to very ususual amino acid, e.g., β-amino acid, 4-methylazetidinecarboxylic acid and homoproline. Approximately 40% of the compounds were absolutely novel structures, while the others were new but contained structural elements which were reported previously (Reichenbach, 2001).

Antibiotic production by myxobacteria was firstly reported in 1947 by Oxford (Arias *et al*., 1979, referred to Oxford, 1947) but no chemical structure was elucidated. Later, Peterson and co-worker (1966) which referred by Arias and others (1979) reported that myxin, an antibiotic produced by *Sorangium* could be detected at the end of the logarithmic phase. Myxin at the concentration of 1 μg/ml had been reported to possess an unusually broad antimicrobial spectrum by the inhibition of deoxyribonucleic acid (DNA) synthesis without affecting the synthesis of either ribonucleic acid (RNA) or protein (Lesley and Behki, 1967).

Arias and colleagues (1979) also reported the fermentation and isolation of an antibiotic substance produced by *Myxococcus coralloides* which capable of inhibiting the growth of Gram-positive bacteria. It was important to state that the antibiotic production occurred only during vegetative growth but not during myxospore formation, usually after 24 hours and increased at a linear rate for the next 36 hours. Maximum antibiotic production occurred between 4 and 6 days whereas growth was decreased after 48 hours. The antibiotic was extracted from the growth medium with chloroform and purified by silicic acid (SIL-R) column and preparative silica gel thin-layer chromatography. The purified antibiotic showed a resistance to heat, acid, alkali and proteolytic enzymes. The partial characterization of this compound was investigated; however, its chemical structure was not proposed (Arias *et al*., 1979).

Myxothiazol (**1** in Fig. 8), a new antifungal antibiotic produced by *Myxococcus fulvus* strain Mx f16, was described by Gerth and colleagues (1980). Fermentation process in order to obtain this compound was performed in 70-liter fermentor containing 63 liters of peptone liquid medium at 30°C, 500 rpm. The red cell mass was separated from the culture broth by centrifugation at the end of fermentation and it was extracted with acetone until the cells were colorless. The red and highly viscous residue was redissolved in methanol and the inactive non-polar materials were removed by partitioning with *n*-heptane/methonal. The methanol phase was then concentrated under reduced pressure and the residue was dissolved in ethyl acetate before extracting twice with 25% aqueous ammonia in order to remove the fatty acids. After neutralization, the ethyl acetate solution was concentrated and the antibiotic was isolated by a column chromatography with silica gel using 1:4 acetone:*n*-heptane as an eluant. The resulting material contained of 95 % myxothiazol. Final purification was achieved by a column chromatography on reversed-phase silica gel RP-18 using 4:1 methanol:water as an eluant. The molecular formula of myxothiazol was determined as $C_{25}H_{33}N_3O_3S_2$. Its biological activities included antimicrobial activity against Gram-positive bacteria and numerous fungi excluding yeasts and Gram-negative bacteria (Gerth *et al*., 1980).

O'Sullivan *et al*. (1988) described a novel antibacterial agent, lysobactin (**2** in Fig. 8), produced by a gliding bacterium *Lysobacter sp*. ATCC53042 using a medium containing yeast extract (0.5%), peptone (0.3%), mannitol (0.5%) and distilled water. The flasks were incubated at 25°C on a rotary shaker (300 rpm) for approximately 24-48 hours. Lysobactin was isolated from both cells and broth. The cell mass was extracted three times with 200-ml of 4:1 acetone:water while fermentation broth was concentrated by extracting the broth supernatant with butanol. Lysobactin was purified by a reversed phase column chromatography and its chemical structure was elucidated by spectroscopic techniques.

Lysobactin was considered as a dibasic peptide with marked activity against Gram-positive bacteria including *Staphylococcus aureus*, *Micrococcus luteus* and *Clostridium difficile* (O'Sullivan *et al*., 1988).

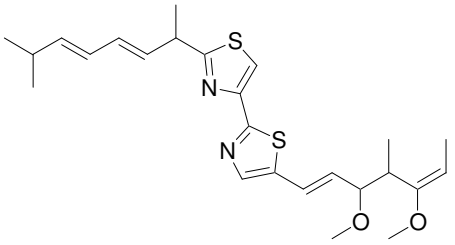
A novel antifungal compounds, soraphen A (**3** in Fig. 8), produced by *Sorangium cellulosum* was reported by Gerth and colleagues (1994). Growth and soraphen A production were very much influenced by the incubation temperature; when temperature was raised from 30 to 40 °C, the generation time increased from 11 to 18 hours, and soraphen A production was completely inhibited. The addition of XAD-1180 resins had no effect on the cell growth but was beneficial to the antibiotic production. In the absence of resin, the yield of soraphens dropped 70%.

Reichenbach (2001) described soraphen's novel mechanism of action on fungal growth by specifically blocking a fungal acetyl-CoA carboxylase. It was once considered to be a potential fungicide but its unacceptable side effects halted the further development.

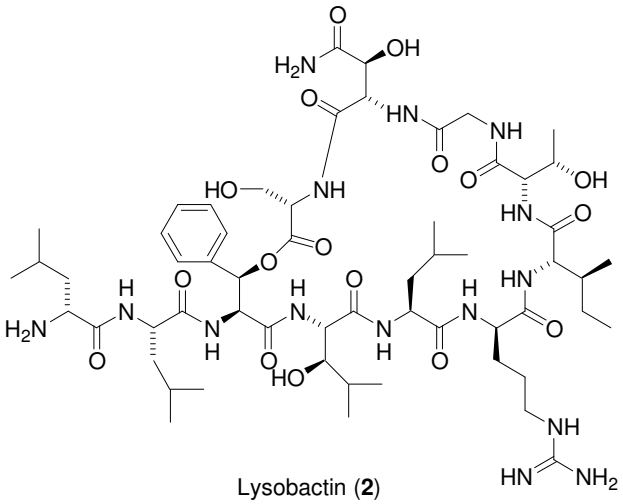
Suzuki *et al*., (1998) reported the structures of new bithiazole antifungal compounds, cystothiazoles (**4a-4c** in Fig. 8); isolated from the myxobacterium *Cystobacter fuscus* strain AJ-13278. The fermentation was performed at 28 °C for 4 days in the presence of an adsorber resin. The bacterial cells and the adsorber resin were separately collected from the 150-liter culture broth and extracted with acetone. Each extract was partitioned with 3:1 hexane-EtOAc and water. The organic fractions from the bacterial cells and resin were combined and separated by a series of silica gel column, ODS column, and HPLC to give cystothiazoles A-F. Cystothiazole C (**4c**) showed similar UV, IR and NMR spectrum to that of cystothiazole A (**4a**), suggesting that the chemical structure of cystothiazole C was closely related with cystothiazole A. Later, Akita *et al*., (2004) proposed the structure of cystothiazole G (**4g**) isolated from *Cystobacter fuscus*. Cystothiazoles showed antifungal activity against the phytopathogenic fungus *Phytophthora capsici* (Akita *et al*., 2004; Suzuki *et al*., 1998). Its mode of action was known to be the inhibition of NADH oxidation in the submitochondrial membrane.

Apicularens A (**5a**) and B (**5b**) (Fig. 8), the new cytostatic macrolides produced by several species of the genus *Chondromyces* (Myxobacteria) were reported by Kunze and co-workers (1998). Apicularen A, obtained as colorless crystals, was soluble in methanol, acetone, chloroform, and ethylacetate whereas an amorphous, more polar apicularen B was very well soluble in water. Apicularens A and B shown Rf values of 0.39 and 0.1, respectively when separated on silica gel 60 TLC plate using 9:1 dichloromethane:methanol as a mobile phase. They could be visualized as brown-pink spots after spraying with vanillin/sulfuric acid reagent and heating at 120 °C. The UV spectra of apicularen A and apicularen B showed the same λ_{max} (logε)

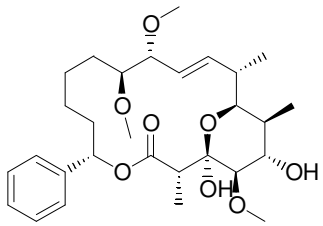
at 278 nm. Apicularen A was completely inactive against a series of bacteria, yeasts and filamentous fungi. Apicularen B showed weak activity against a few Gram-positive bacteria i.e. *Micrococcus luteus* and *Corynebacterium fascians* with the MICs of 12.5 and 25 μg/ml, respectively. On the other hand, apicularen A showed potent cytotoxicity with the IC_{50} value of 1 ng/ml (Kunze *et al.*, 1998).



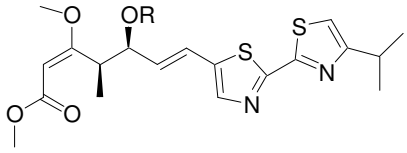
Myxothaiazol (**1**)



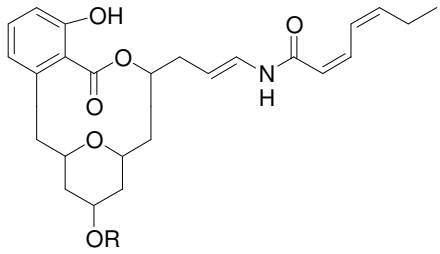
Lysobactin (**2**)



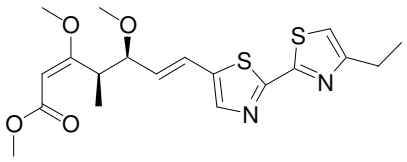
Soraphen A (**3**)



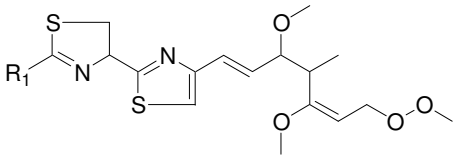
Cystothiazole A (**4a**), R = CH_3
Cystothiazole C (**4c**), R = H



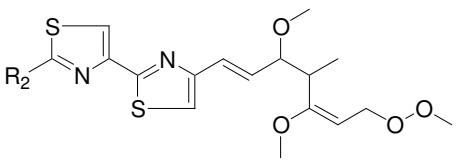
Apicularen A (**5a**), R = H
Apicularen B (**5b**), R = *N*-acetyl-beta-D-glucosamide



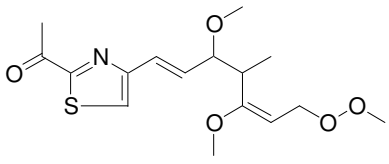
Cystothiazole G (**4g**)



Melithiazol A (**6a**), R_1 = $C(CH_3)=CH_2$
Melithiazol D (**6d**), R_1 = $CH(CH_3)_2$
Melithiazol L (**6l**), R_1 = $C(CH_3)CH_2OCH_3$



Melithiazol B (**6b**), R_2 = $C(CH_3)=CH_2$
Melithiazol E (**6e**), R_2 = $CH(CH_3)_2$
Melithiazol F (**6f**), R_2 = $CH_2C_6H_6$
Melithiazol G (**6g**), R_2 = $CH(CH_3)CH_2CH_3$
Melithiazol H (**6h**), R_2 = C_2H_5
Melithiazol I (**6i**), R_2 = $CH_2CH(CH_3)_2$
Melithiazol M (**6m**), R_2 = $C(O)CH_3$



Melithiazol C (**6c**)

Figure 8. Strucutres of myxothiazol, lysobactin, soraphen A, cystothiazoles, apicularens and melithiazols

Sources: Gerth *et al*. (1980); Kunze *et al.* (1998); Sasse *et al*. (1999); Suzuki *et al*. (1998)

Sasse and colleagues (1999) reported the physico-chemical of the new antibiotic compounds, methiazols (**6a-6i, 6l, 6m** in Fig. 8), isolated from *Melittangium lichenicola*, *Archangium gephyra*, and *Myxococcus stipitatus*. Methiazols were soluble in methanol, acetone, ethylacetate, and chloroform. The Rf values of methiazols A (**6a**) and B (**6b**), with 9:1 dichloromethane:acetone as the solvent, were 0.55 and 0.69, respectively. Melithiazols had no antibacterial activity but it showed antifungal activity against *Hansenula anomala*, *Metschnikowia pulcherrima*, *Botrytis cinerea* and *Pythium debaryanum*. Mode of action of the melithiazols was the inhibition of NADH oxidation whereas melithiazols A could also block the electron transport within the bc_1-segment (complex III) and caused a red shift in the reduced spectrum of cytochrome *b* (Sasse *et al*., 1999).

New cytostatic compounds, tubulysins (**7a-7b, 7d-7e** in Fig. 9), isolated from the culture broth of myxobacteria, *Archangium gephyra* and *Angiococcus disciformis*, were proposed by Sasse *et al*. (2000). Tubulysins were soluble in methanol, acetone, and ethyl acetate. The antimicrobial activities of the tubulysins A (**7a**) and B (**7b**) were determined by an agar diffusion assay. Both tubulysins A and B were completely inactive against bacteria and yeasts with only weak antifungal activity against some filamentous fungi. Tubulysins A, B, D (**7c**), and E (**7d**) were highly cytotoxic, compared to dolastatin-10, with IC_{50} values ranging from 1 ng/ml to 20 pg/ml. Tubulysins D showed 10 times higher activity than tubulysins A and dolastatin-10. Their kinetic properties showed that the propagations of all tested cell lines (L-929, PtK_2, KB-3.1, KB-V1 and K-562) were inhibited immediately after the addition of the tubulysins. Although the cells division was inhibited but cell growth was not totally inhibited. Based on this, it was suggested that tubulysins could be developed as antitumor drugs (Sasse *et al.*, 2000).

The mechanisms of action of myxobacterial fungicide Ambruticin were studied by Ambruticins S (**8a**) and VS-3 (**8b**) (Fig. 9), novel antifungal compound, were isolated from a myxobacterium *Sorangium cellulosum* (Knauth and Reichenbach, 2000). Ambruticins were active against various pathogenic yeasts and fungi including *Trichophyton* and *Microsporum* species, *Histoplama capsulatum*, *Coccidioides immitis*, *Blastomyces dermatididis* and *Aspergillus* spp. The MIC of ambruticin VS-3 against *Hansenula anomala* was 0.05 μg/ml. (Knauth and Reichenbach, 2000).

Approximately 10% of cytotoxic metabolites from myxobacteria interact specifically with the cytoskeleton of eukaryotic cells, indicating high potential for the development of anticancer drugs. Around 10 different novels basic structures of these cytotoxic compounds affected specifically on actin, tubulin, and cytoskeleton and induced apoptosis. Rhizopodin (**9** in Fig. 9), was first to be discovered from the culture broth of *Myxococcus stipitatus*, showed the induction of L929 mouse fibroblasts to develop long, thin, branched extensions, or runners, resembling the rhizopodia of certain protozoa with the minimal dose (Reichenbach, 2001).

Chondramide A (**10** in Fig. 9), isolated from *Chondromyces crocatus*, is showed activity on actin stabilization. Mechanism of action of chondramide appeared to have the same binding site on actin as did phalloidin which was a toxin produced by green and white deathcaps. In contrast to phalloidin, however, chondramide A penetrated all kinds of mammalian cells and the IC_{50} values of chondramide A for different tumor cell lines were ranging between 3 and 85 nM (Reichenbach, 2001).

Among myxobacterial secondary metabolites, epothilones A (**11a**) and B (**11b**) (Fig. 9) probably attracted highest interest in drug discovery because of its paclitaxel like activity in stabilizing microtubule causing apoptosis. Epothilon also killed multidrug-resistant cancer cells, including paclitaxel-resistant. Because of its high solubility in water and possibility in production by fermentation process, it becomes a promising candidate for an antitumor drug (Reichenbach, 2001).

Vollbrecht *et al*. (2002) isolated cyclic octapeptides, argyrins A-H (**12a-12h** in Fig. 9). Argyrins A (**12a**) and B (**12b**) contain two unusual amino acids, 2-(12-aminoethyl) thiazol-4-caboxylic acid and the novel amino acid 4′-methoxytryptopha in addition to tryptophan, glycine, dehydroalanine and alanine or α-aminobutyric acid, sarcosine. According to their NMR spectra, argyrins A and B were identical in $CDCl_3$ and slightly different in acetone-d_6.

Jansen and *et al*. (2003) reported the discovery of new β-amino styrenes, chondrochlorens A (**13a**) and B (**13b**) (Fig. 9), from the myxobacteria *Chondromyces crocatus*. Chondrochloren B contained an additional methylene group, replacing the methoxy group at C-2 of chondrochloren A.

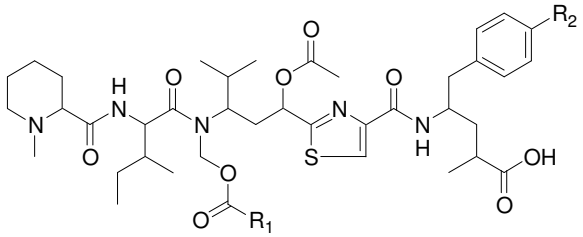
Chondrochlorens showed weak antibiotic activity against *Micrococcus luteus*, *Schizosaccharomyces bombe*, *Bacillus subtilis* and *Staphylococcus aureus* (Jansen *et al.*, 2003).

Myxalamides (**14a-14c**) (Fig. 10), novel antifungal polyene amides, produced by a myxobacterium *Cystobacter fuscus* were proposed by Kundim *et al*. (2004). The antifungal activity of myxaiamides was evaluated by a paper disc diffusion assay against the phytopathogenic fungus *Phytophthora capsici*, showing the activity at the concentration of 2 µg/disc. Since all three isomers showed the same antifungal activity against the fungus *P. capsici*, it was suggested that the olefin geometry played no crucial role for their antifungal activity (Kundim *et al*., 2004).

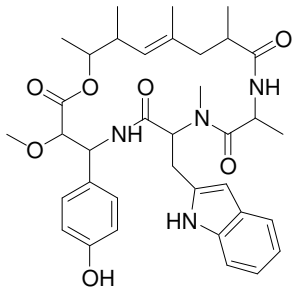
Kunze *et al*. (2005) reported the new antibiotics, aurafurons A (**15a**) and B (**15b, 15c**) (Fig. 10), from myxobacteria *Archangium gephyra* and *Stigmatella aurantiaca*, respectively. Fermentation of *Archangium gephyra* was performed in modified M7 liquid medium (Probion 0.5%, starch 0.5%, glucose 0.2%, yeast extract 0.1%, $MgSO_4.7H_2O$ 0.1%, $CaCl_2.2H_2O$ 0.1%, cyanocobalamine 0.1 mg/liter, 10 mM HEPES buffer 1.0%, pH 7.0) with the addition of 1% of the adsorber resin Amberlite XAD-16 yielding 200 mg of aurafurons A. Fermentation of *Stigmatella aurantiaca* was performed in 150 liters bioreactors containing 85 liters Zein liquid medium (Zein 0.8%, peptone 0.1%, $MgSO_4.7H_2O$ 1.0%, 10 mM HEPES buffer, pH 7.2) with 1% of the adsorber resin Amberlite XAD-16, yielding 16 mg of aursfuron isomer B1 (**15b**), and 2 mg isomer B2 (**15c**).

Aurafurons inhibited the growth of some filamentous fungi with weak activity against a few Gram-positive bacteria. Aurafurons showed cytotoxic activity against the mouse fibroblast with the IC_{50} of aurafuron A at 4 µg/ml and aurafuron B1 at 0.35 µg/ml (Kunze *et al*., 2005).

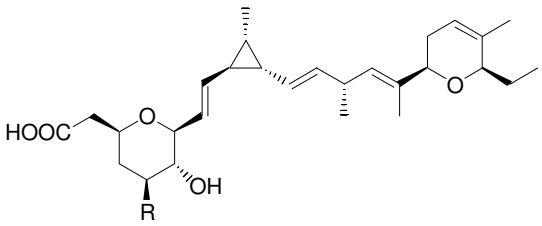
Ahn and colleagues (2007) reported the isolation of new bithiazole metabolites from myxobacterium *Myxococcus fulvus*. Bithiazoles A (**16a**) and B (**16b**) (Fig. 10) exhibited significant cytotoxicity against the mouse fibloblast cell-line L929 and moderate antifungal activity against *Candida albicans* (Ahn *et al*., 2007).



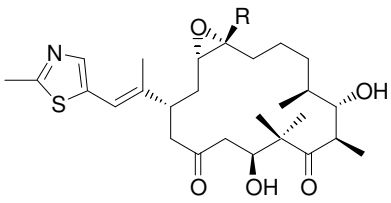
Tubulysin A (**7a**), $R_1 = CH_2CH(CH_3)_2$; $R_2 = OH$
Tubulysin B (**7b**), $R_1 = CH_2CH_2CH_3$; $R_2 = OH$
Tubulysin D (**7d**), $R_1 = CH_2CH(CH_3)_2$; $R_2 = H$
Tubulysin E (**7e**), $R_1 = CH_2CH_2CH_3$; $R_2 = H$



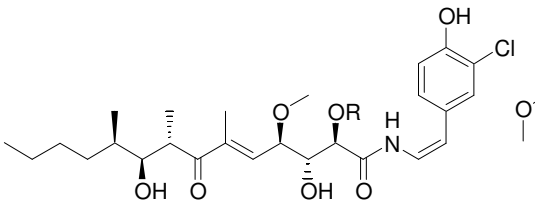
Chondramide A (**10**)



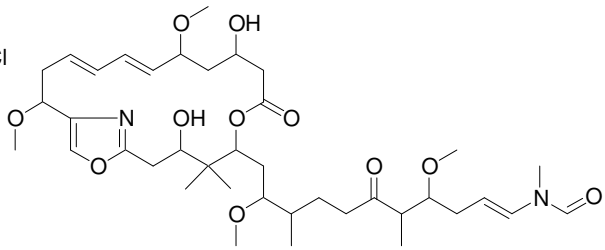
Ambruticin S (**8a**), R = OH
Ambruticin VS-3 (**8b**), $R = NH(CH_3)_2^+$



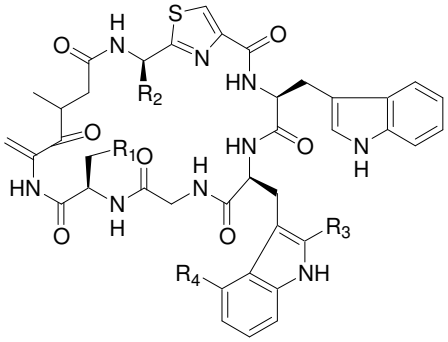
Epothilon A (**11a**), R = H
Epothilon B (**11b**), $R = CH_3$



Chondrochloren A (**13a**), $R = CH_3$
Chondrochloren B (**13b**), R = Et



Rhizopodin (**9**)

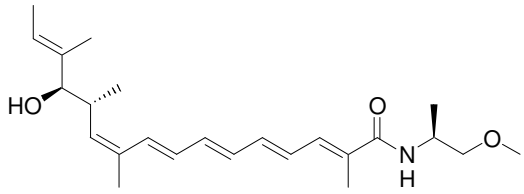


Argyrin A (**12a**), $R_1 = H$, $R_2 = CH_3$, $R_3 = H$, $R_4 = OCH_3$
Argyrin B (**12b**), $R_1 = CH_3$, $R_2 = CH_3$, $R_3 = H$, $R_4 = OCH_3$
Argyrin C (**12c**), $R_1 = H$, $R_2 = CH_3$, $R_3 = CH_3$, $R_4 = OCH_3$
Argyrin D (**12d**), $R_1 = CH_3$, $R_2 = CH_3$, $R_3 = CH_3$, $R_4 = OCH_3$
Argyrin E (**12e**), $R_1 = H$, $R_2 = CH_3$, $R_3 = H$, $R_4 = H$
Argyrin F (**12f**), $R_1 = H$, $R_2 = CH_2OH$, $R_3 = H$, $R_4 = OCH_3$
Argyrin G (**12g**), $R_1 = CH_3$ $R_2 = CH_2OH$, $R_3 = H$, $R_4 = OCH_3$
Argyrin H (**12h**), $R_1 = H$, $R_2 = H$, $R_3 = H$, $R_4 = OCH_3$

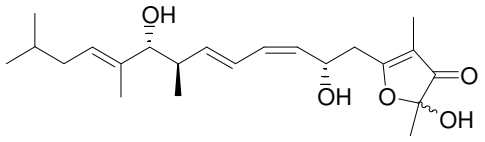
Figure 9. Structures of tubulysins, ambruticins, rhizopodin, chondramide A, epothilons, argyrins and chondrochloren

Sources: Jansen *et al.* (2003); Reichenbach (2001); Vollbrecht *et al*. (2002)

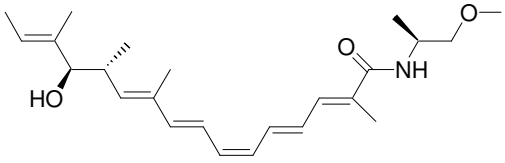
Soraphinol B (**17** in Fig. 10) a new acyloin compound produced by *Sorangium cellulosum* was also reported by Ahn *et al*. (2007). The IC_{50} value of soraphinol B against protein farnesyl transferase (PFTase) was 15.1 µg/ml.



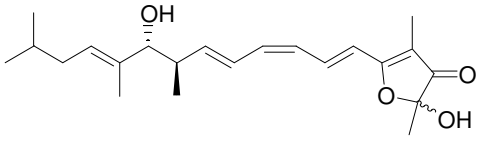
(6*E*, 10*Z*)-2'-*O*-methylmyxalamide D (**14a**)



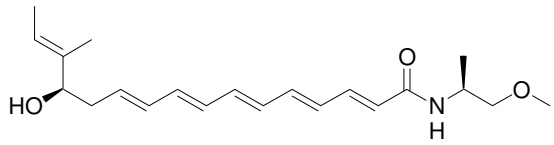
Aurafuron A (**15a**)



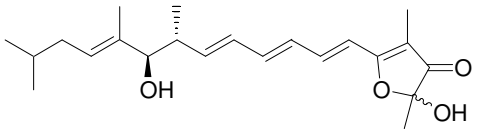
2'-*O*-methylmyxalamide D (**14b**)



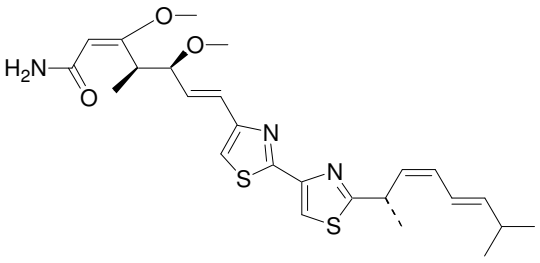
Aurafuron B1 (**15b**)



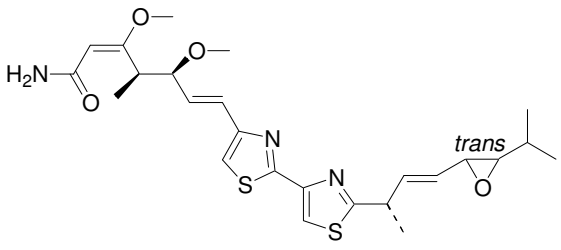
(6*E*)-2'-*O*-methylmyxalamide D (**14c**)



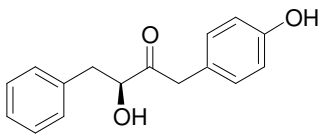
Aurafuron B2 (**15c**)



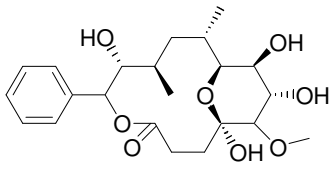
Bithiazole A (**16a**)



Bithiazole B (**16b**)



Soraphinol B (**17**)



Phoxalone (**18**)

Figure 10. Structures of methylmyxalamides, aurafurons, bithiazoles, soraphinol B and phoxalone
Sources: Ahn *et al*. (2007); Kundim *et al*. (2004); Kunze *et al*. (2005)

Phoxalone (**18** in Fig. 10), a novel macrolide produced from *Sorangium cellulosum* WXNXJ-C was proposed by Guo and Tao (2008). *Sorangium cellulosum* WXNXJ-C was cultivated in seed medium (potato starch 10 g/l, glucose 2 g/l, defatted soybean meal 4 g/l, yeast extract 2 g/l, Fe(III)-Na-EDTA 0.008 mg/l, $CaCl_2.2H_2O$ 1 g/l, $MgSO_4.7H_2O$ 1 g/l, HEPES 11.5 g/l, pH 7.5) at 30 °C, 160 rpm for 3 days before transferring into fermentation medium (potato starch 10 g/l, glucose 2 g/l, defatted soybean meal 4 g/l, yeast extract 2 g/l, Fe(III)-Na-EDTA 0.008 mg/l, $CaCl_2.2H_2O$ 1 g/l, $MgSO_4.7H_2O$ 1 g/l, pH 7.5). Phoxalone exhibited strong inhibitory activity against tumor cell lines i.e. B16, Bel7402, H446, MCF-7, and SGC7901. The IC_{50} values indicated that phoxalone has the higher inhibition to B16 (IC_{50} 0.5 μg/ml) and MCF-7 (IC_{50} 1 μg/ml) cell than SGC7901 cell line (IC_{50} 4 μg/ml). Moreover, phoxalone showed the apoptosis induction of H446 cell line. A cytotoxic bioactivity on H446 cell line *in vitro* suggested that phoxalone arrested the mitosis in the G2/M phase with cytotoxicity to normal human liver L02 cell lines (286 μg/ml) (Guo and Tao, 2008).

Because many secondary metabolites produced by gliding bacteria were detected in the early log phase instead of late stationary phase like other bacterial secondary metabolites, suggested that this might be due to the very short stationary phase of myxobacteria. For instance, this phenomenon was quite dramatic in the case of *Stigmatella aurantiac* when cultivated in a peptone medium. Within 2 h after stationary phase was reached, the cells lysed and the medium became pitch black because of its secondary metabolites, usually myxovirescin. Most myxobacterial compounds were excreted into the medium and several reports indicated that the use of an amberlite type resin, e.g., XAD-16 was found to be very useful for absorbing the compounds from small scale to industrial scale fermentation. The resins were added to remove the compounds from reaching the equilibrium, therefore, preventing end product inhibition, modification and degradation of the compounds (Reichenbach, 2001).

Other bioactive compounds produced from myxobacteria are summarized in Table 2.

Table 2. List of bioactive compounds produced by myxobacteria

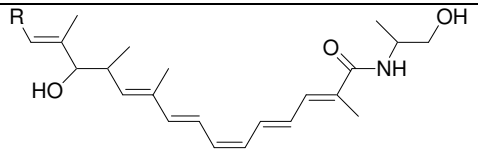
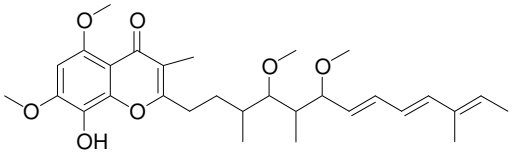
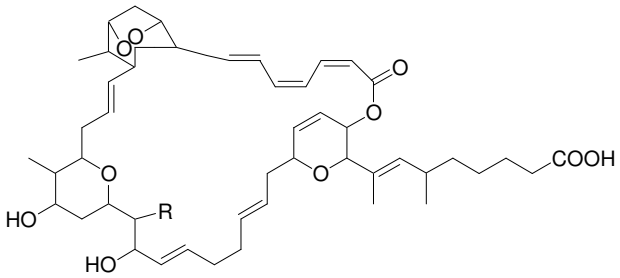
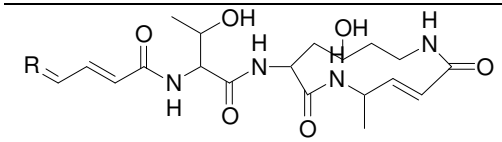
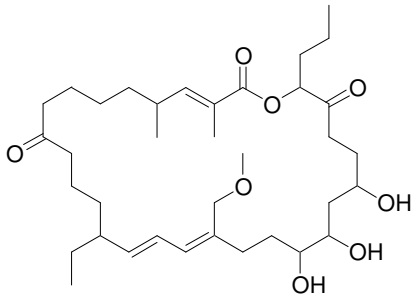
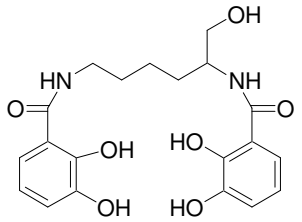
Compound	Producing species	Biological activity	Reference
 Myxalamid A (**19a**), R = $CH_3CH_2(CH_3)CH$ Myxalamid B (**19b**), R = $(CH_3)_2CH$ Myxalamid C (**19c**), R = CH_3CH_2 Myxalamid D (**19d**), R = CH_3	*Myxococcus xanthus*	Cytotoxic Antifungal Antibacterial	Gerth *et al*. 1983
 Stigmatellin (**20**)	*Stigmatella aurantiaca*	Antibacterial Antifungal	Kunze *et al*. 1984
 Sorangicin A (**21a**), R = OH Sorangicin B (**21b**), R = H	*Sorangium cellulosum*	Antibacterial Antifungal	Irschik *et al*. 1987

Table 2. List of bioactive compounds produced by myxobacteria (Cont.)

Compound	Producing species	Biological activity	Reference
 Glidobactin A (**22a**), R = $CH_3(CH_2)_6$ Glidobactin B (**22b**), R = $CH_3(CH_2)_4CH{=}CH(CH_2)_2$ Glidobactin C (**22c**), R = $CH_3(CH_2)_8$	*Polyangium brachysporum*	Antitumor	Oka *et al*. 1988
 Megovalicin B (**23**)	*Myxococcus flavescens*	Antibacterial	Takayama *et al*. 1988
 Myxochelin A (**24**)	*Angiococcus disciformis*	Antibacterial	Kunze *et al*. 1989

1.2.4 Bioactive compounds produced by CFB

A quinoline compound, G1499-2 (**25** in Fig. 11), produced by *Cytophaga johnsonii* ATCC 21123, was reported by Evans and Napier (1978). Compound G1499-2 showed an unusual structure containing a cyclopropylidene radical. The physical and chemical measurements of crystalline G1499-2 suggested the novel structure 3-methyl-2-(2-pentylidenecyclopropyl)-4-quinolinone. G1499-2 inhibited the growth of *Staphylococcus aureus* and *Bacillus subtilis* but did not inhibit the growth of yeasts and fungi (Evans and Napier, 1978).

Katayama *et al*. (1985) reported the discovery of new monocyclic β-lactam antibiotics, formadicins (**26a-26d** in Fig. 11), from *Flexibacter alginoliquefaciens* sp. nov. YK-49 when cultivated in medium consisting of glycerol 3 %, soybean flour 2 %, corn gluten meal 1 %, polypepton 0.2 %, $CaCO_3$ 0.5 % and actocol 0.05 % in a 2,000-liter fermentor. The cultivation was carried out at 20°C for 66 hours with aeration (1,200 liters/minute) and agitation rate at 120 rpm.

Formadicins showed comparatively narrow antibacterial activity against *Pseudomonas*, *Proteus*, and *Alcaligenes*. Formadicins C (**26c**) and D (**26d**) were more active than formadicins A (**26a**) and B (**26b**) (Fig. 11). Formadicins A and C showed fairly good protective effect in mice from intraperitoneal infection by *E. coli* and *P. vulgalis* and no acute toxicity of these compounds was observed in mice (Katayama *et al*., 1985).

Monobactam PB-5266 A (**27a**), B (**27b**) and C (**27c**) (Fig. 11) isolated from the culture filtrate of *Cytophaga johnsonae* P5266 were proposed by Kato *et al.* (1987). The fermentation was performed in 20 liters of a production medium consisting of fish meal 2.0%, potato starch 1.0%, corn starch 1.0%, KH_2PO_4 0.5% and adjusted to pH 4.5 with H_3PO_4. Fermentation was carried out at 28°C for 1 day under aeration of 20 liters per minute and agitation of 200 rpm. These compounds exhibited weak antibacterial activity against a β-lactam sensitive mutant *Escherichia coli* (Kato *et al*., 1987).

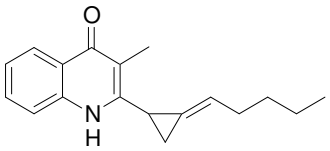
Two peptide antibiotics, katanosins A (**28a**) and B (**28b**) (Fig. 11), isolated from the culture broth of *Cytophaga* sp. PBJ-5356 were reported by Shoji *et al*. (1988). The authors described that the crystals, obtained from acidifying of the methanol fraction of the crude extract with 1N HCl, were shown to be a complex of katanosins A and B in the form of hydrochlorides. The molecular formulae of katanosins A and B were $C_{57}H_{95}N_{15}O_{17}$ and $C_{58}H_{97}N_{15}O_{17}$, respectively. The constituent amino acids of katanosin A are Thr (1), Ser (1),Val (1), Leu (3), Arg (1) and three unusual amino acids which in katanosin B, the Val residue is replaced by Ile. Both compounds showed antibacterial activity against Gram-positive bacteria i.e *Staphylococcus aureus*, *Streptococcus pyogenes*, *Streptococcus pneumoniae* and *Streptococcus faecalis* both *in vitro* and *in vivo* (Shoji *et al*., 1988).

Topostins (**29a, 29b** in Fig. 11) produced by *Flexibacter topostinus* sp. nov. were reported by Suzuki *et al*. (1990). These compounds exhibited the inhibitory effect mammalian DNA topoisomerase I (Suzuki *et al.*, 1990).

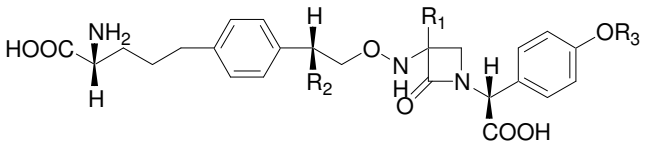
Resorcinin (**30** in Fig. 11), a mammalian cell growth stimulating substance produced by *Cytophaga johnsonae* was reported by Imai *et al*. (1993). Resorcinin exhibited growth-stimulating activity to NIH 3T3 mouse fibroblast cells in the presence of only 1% of fetal calf serum (FCS) which normally did not grow without the addition of resorcinin. However, resorcinin also showed the cytotoxicity against mammalian cells (P388 murine leukemia) with the IC_{50} of 9.8 µg/ml (Imai *et al*., 1993).

New anti-staphylococcal antibiotics, TAN-1057 A (**31a**) and B (**31b**) (Fig. 11), were discovered by Katayama *et al*. (1993). These antibiotics were isolated from the culture filtrates of *Flexibacter* sp. PK-74 and PK-176. TAN-1057 showed stronger antibacterial activity against Gram-positive bacteria i.e. *Staphylococcus aureus*, *S. epidermidis*, *Bacillus subtilis*, *B. cereus* than Gram-negative bacteria i.e. *Escherichia coli*, *Serratia marcescens*, *Pseudomonas aeruginosa*, *Proteus vulgaris*. TAN-1057A inhibited the incorporation of leucine into macromolecules in *E. coli* and *S. aureus* at the MIC of 12.5 µg/ml and 3.13 µg/ml, respectively. Moreover, it inhibited poly-A and poly-U directed peptide synthesis in the *E. coli* cell-free system, with the IC_{50} of 10 µg/ml and 40 µg/ml, respectively. TAN-1057A showed an extremely strong therapeutic effect against experimental infection with *S. aureus*, including methicillin-resistant

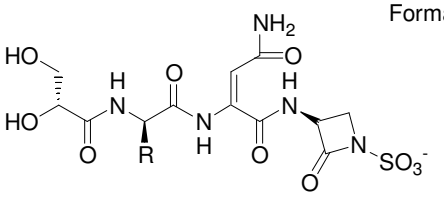
strains (MRSA), in mice. The preliminary acute toxicity (LD_{50}) of TAN-1057 A in mice was 100 mg/kg upon intra-peritoneal and 50 mg/kg upon intravenous injection (Katayama *et al*., 1993).



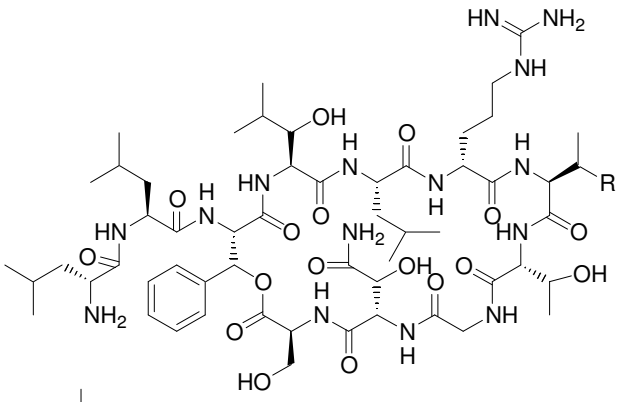
G1499-2 (**25**)



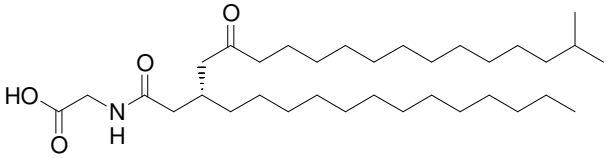
Formadicin A (**26a**), R_1 = NHCHO, R_2 = OH, R_3 = D-glucuronic acid
Formadicin B (**26b**), R_1 = H, R_2 = NHCHO, R_3 = D-glucuronic acid
Formadicin C (**26c**), R_1 = NHCHO, R_2 = OH, R_3 = H
Formadicin D (**26d**), R_1 = H, R_2 = NHCHO, R_3 = H



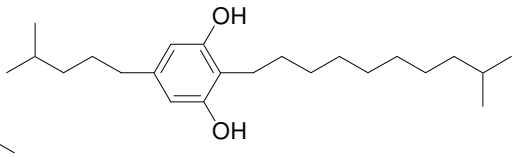
PB-5266A (**27a**), R = CH_3
PB-5266B (**27b**), R = CH_2OH



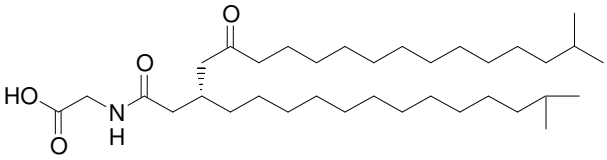
Katanosin A (**28a**), R = CH_3
Katanosin B (**28b**), R = CH_2CH_3



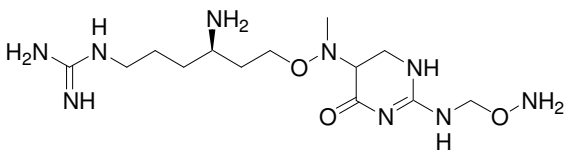
Topostins B553 (**29a**)



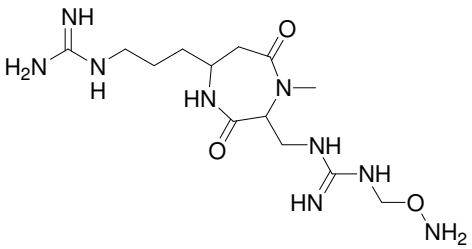
Resorcinin (**30**)



Topostins B567 (**29b**)



TAN-1057A (**31a**)



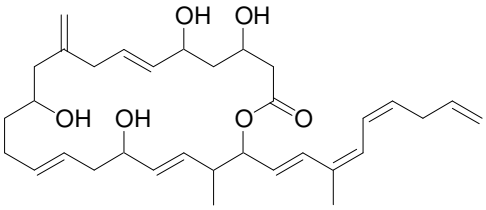
TAN-1057B (**31b**)

Figure 11. Structures of G1499-2, formadicins, PB-5266, katanosin, topostins, resorcinin and TAN-1057

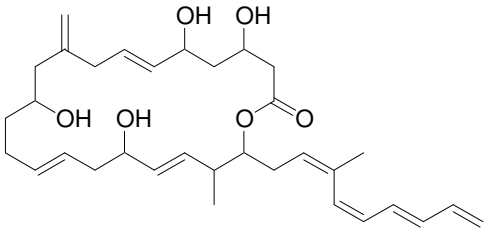
Sources: Evans and Napier (1978); Imai *et al*. (1993); Katayama *et al*. (1985); Katayama *et al*. (1993); Kato *et al.* (1987); Shoji *et al*. (1988); Suzuki *et al*. (1990)

Kamigiri and co-workers (1997) proposed the new 22-membered macrolide antibiotics, YM-32890 A (**32a**) and B (**32b**) (Fig. 12) isolated from the culture broth of *Cytophaga* sp. strain YL-02905S. YM-32890 A exhibited a potent antibacterial activity against *Staphylococcus aureus* and *S. epidermidis* but showed no antimicrobial activity against other Gram-positive, Gram-negative bacteria and yeasts. YM-32890 B, on the other hand, did not show any antimicrobial activity. Cytotoxicty of YM-32890 A and B against L1210 cells were 15.7 and 70.0 µg/ml respectively. It was suggested that the side chain structure in YM-32890 A played an important role in anti-staphylococcal activity (Kamigiri *et al*., 1997).

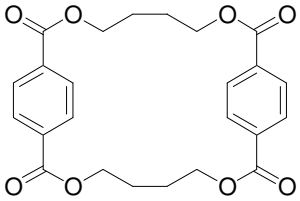
Pharacine (**33** in Fig. 12) a natural *p*-cyclophane and 2,5-bis(3-indolylmethyl)pyrazine (**34** in Fig. 12) and other known indole derivatives (**35-52** in Fig. 12) isolated from *Cytophaga* sp. strain AM13.1 were proposed by Shaaban *et al*. (2002). Pharacine and 2,5-bis(3-indolylmethyl)pyrazine, showed no activity against microalgae*, Mucor miehei*, *Candida albicans*, *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Streptomyces viridochromogenes* whereas tryptanthrin (**35**) showed antibacterial and antifungal activities against *Bacillus subtilis* and *Mucor miehei*, respectively. It was suggested that phenylethyl amides were also responsible for the weak phycotoxicity against the microalgae *Chlorella vulgaris*, *Chlorella salina* and *Scenedesmus subspicatus* (Shaaban *et al*., 2002).



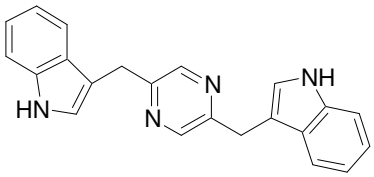
YM-32890 A (**32a**)



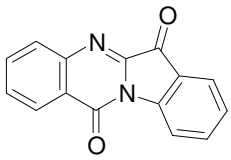
YM-32890 B (**32b**)



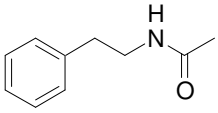
Pharacine (**33**)



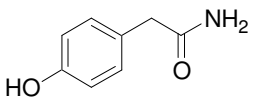
2,5-bis(3-indolymethyl)pyrazine (**34**)



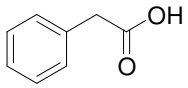
Tryptanthrin (**35**)



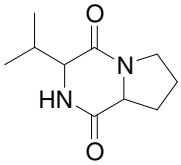
2-phenylethylacetamide (**36**)



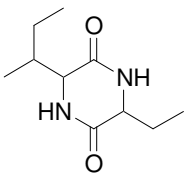
p-hydroxyphenylacetamide (**37**)



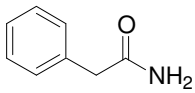
p-phenylacetic acid (**38**)



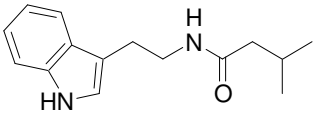
Cyclo(valylprolyl) (**39**)



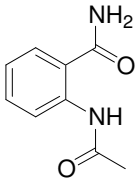
Cyclo(isoleucylvalyl) (**40**)



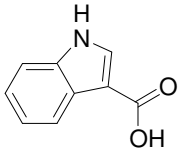
Phenylacetamide (**41**)



Tryptamine isolerate (**42**)



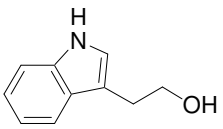
O-acetylaminobenzamide (**43**)



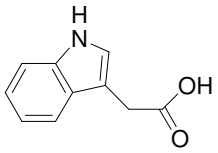
3-indolyl-carboxylic acid (**44**)

Figure 12. Structures of bioactive compounds produced by *Cytophaga* sp., YM-32890, pharacine, 2,5-bis(3-indolymethyl) pyrazine, tryptanthrin, 2-phenylethylacetamide, p-hydroxyphenyl acetamide, *p*-phenylacetic acid, cyclo (valylprolyl), cyclo (isoleucylvalyl), phenylacetamide, tryptamine isolate, *o*-acetylaminobenzamide and 3-indolyl-carboxylic acid

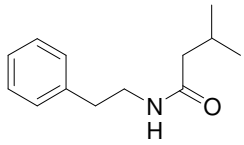
Sources: Kamigiri *et al*. (1997); Shaaban *et al*. (2002)



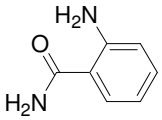
2-(3-indolyl)ethanol (**45**)



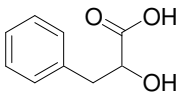
3-indolyl-acetic acid (**4**6)



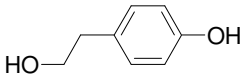
2-phenylethylisovaleramide (**4**7)



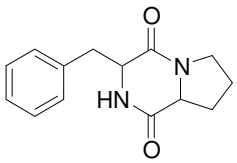
O-aminobenzamide (**48**)



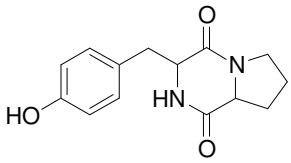
3-phenyllactic acid (**49**)



2-(*p*-hydroxyphenyl)ethanol (**50**)



Cyclo(phenylalanylprolyl) (**51**)



Cyclo(tyrosylprolyl) (**52**)

Figure 12. Structures of bioactive compounds produced by *Cytophaga* sp. (Cont.) 2-(3-indolyl) ethanol, 3-indolyl-acetic acid**,** 2-phenylethylisovaleramide, *o*-aminobenzamide, 3-phenyllactic acid, 2-(*p*-hydroxyphenyl)ethanol, cyclo(phenylalanylprolyl) and cyclo(tyrosylprolyl)

Sources: Shaaban *et al*. (2002)

1.2.5 Bioactive compounds produced by marine gliding bacteria

In general, almost all of secondary metabolites produced by gliding bacteria came from the only one taxonomic group, Myxobacteria. Only a few reports on secondary metabolites from non-myxobacteria have been made so far. It may be possible to assume that gliding bacteria especially those obtained from marine environment are still under studied in terms of their secondary metabolites.

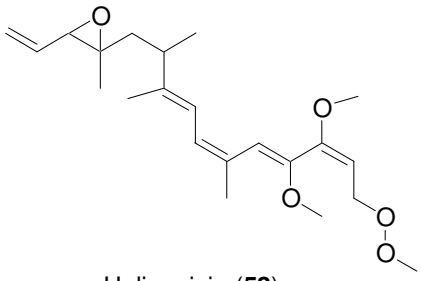
Fudou *et al*. (2001) reported the first antibiotic, haliangicin (**53** in Fig. 13), isolated from a novel marine myxobacterium *Haliangium luteum* sp. nov. obtaining from sandy beach in the Miura peninsula, Kanagawa, Japan (Fudou *et al*., 2001a). The structure of haliangicin was elucidated by HR-FABMS and NMR data (Fudou *et al*., 2001b). It was interesting to note that *H. luteum* required 2-3% NaCl, which are equivalent to the salt concentration seawater, for growth and production of haliangicin. Haliangicin showed potent antifungal activities, comparable to amphotericin B and nystatin, against filamentous fungi. However, haliangicin did not posses any antibacterial activity and the authors suggested that haliangicin blocked the electron flow within cytochrome *b*-*c*1 segment of the respiratory chain of fungi. In addition, haliangicin was found to be toxic to the animal cell line and the IC_{50} against mouse P388 cell was 0.21 μM (Fudou *et al.*, 2001a).

Spyere *et al*. (2003) proposed new neoverrucosane diterpenoids (**54a-54f** in Fig. 13), produced by a marine gliding bacterium *Saprospira grandis*. Unfortunately, the biological activity of these compounds was not evaluated by the authors.

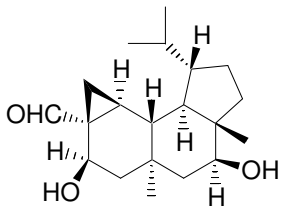
Iizuka *et al*. (2006) reported the isolation of novel antibiotics miuraenamides A (**55a**) and B (**55b**) (Fig. 13), produced from a novel myxobacterium *Paraliomyxa miuraensis* which was isolated from a nearshore soil in Miura peninsula, Japan.

Miuraenamide A exhibited a potent and selective inhibition against a phytopathogenic fungus *Phytophthora* sp. and moderate inhibition against some fungi and yeasts but was inactive against bacteria. Both metabolites inhibited NADH oxidase with the IC_{50} value of 50mM suggesting the β-methoxyacrylate-like activity which electron transfer system of the mitochondrial respiratory chain was the cellular target (Iizuka *et al.*, 2006).

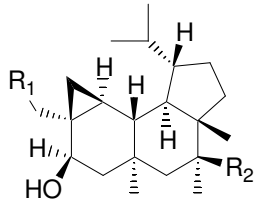
Kanjana-Opas *et al*. (2006) reported the isolation of new marine natural product, marinoquinoline A (**56** in Fig. 13), produced by a novel marine gliding bacterium isolated from southern coast of Thailand. Colourless needle-shaped single crystal of marinoquinoline A was obtained by recrystallization from an acetone-chloroform-hexane (1:1.5:1.5) mixture after 5-7 days and submitted to x-ray diffraction analysis. Marinoquinoline A exhibited a strong inhibitory activity against acetylcholinesterase enzyme with no cytotoxicity against cancer cell lines including HeLa, HT-29, KB and MCF-7 and weak antibacterial activities.



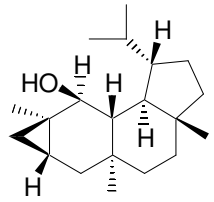
Haliangicin (**53**)



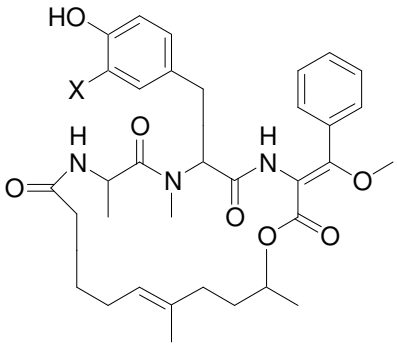
Neoverrucosane diterpenoid 4 (**54d**)



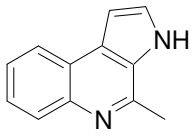
Neoverrucosane diterpenoid 1 (**54a**), R_1 = H, R_2 = OH
Neoverrucosane diterpenoid 2 (**54b**), R_1 = OH, R_2 = H
Neoverrucosane diterpenoid 3 (**54c**), R_1 = R_2 = OH
Neoverrucosane diterpenoid 5 (**54e**), R_1 = R_2 = H



Neoverrucosane diterpenoid 6 (**54f**)



Miuraenamide A (**55a**), X = Br
Miuraenamide B (**55b**), X = I



Marinoquinoline A **(56**)

Figure 13. Structures of bioactive compounds produced from marine gliding bacteria

Sources: Fudou *et al*. (2001); Iizuka *et al*. (2006); Kanjana-Opas *et al*. (2006); Spyere *et al*. (2003)

1.3 Objectives

The objectives of this study are

(i) To isolate the marine gliding bacteria from marine habitats in Thailand

(ii) To identify isolated marine gliding bacteria by 16S rRNA gene sequence analysis

(iii) To screen isolated marine gliding bacteria producing cytotoxic compounds against MCF-7, HeLa, HT-29 and KB cell lines

(iiii) To elucidate the chemical structures of cytotoxic compounds produced by selected marine gliding bacteria

CHAPTER 2

MATERIALS AND METHODS

2.1 General

Unless stated otherwise, all culturation media were commercial grade. The genomic DNAs were extracted by Wizard® genomic DNA purification kit (Promega) and their 16S rRNA fragment was amplified by PCR (GeneAmp® PCR System 9700, Applied Biosystem, Foster City, CA, USA and GeneAmp PCR System 2400, PERKIN ELMER, Norwalk CT, USA). The amplified products were purified by GFX PCR DNA and gel band purification kits (Amersham Biosciences, Buckinghamshire, England) and PCR products were directly sequenced by the ABI PRISM™ BigDye terminator cycle sequencing ready reaction kit version 3.1 (Applied Biosystem, Foster City, CA, USA). Sequencing reactions were carried out either with an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA) or Perkin Elmer 377 sequencer (Macrogen Incorporation, Korea). Amberlite XAD-16 resins used for adsorption of secondary metabolites during cultivation of gliding bacteria were purchased from Fluka Co. Ltd.

The solvents for extraction and column chromatography were both commercial and analytical grades, whereas all HPLC solvents were HPLC grade. The HPLC solvents were filtered through membrane filter (0.45 μm) and were degassed in ultrasonic bath (30 min) prior use.

Size exclusion chromatographic separation was performed using Sephadex® LH-20 (Amersham, Biosciences) as a stationary phase. Preparative HPLC was performed on a Water® 600E multisolvent delivery system and preparative HPLC column (250×10 mm, Luna 100 reversed-phase C-18, 10 μm, Phenomenex) with a Water® 484 tunable detector, and a Rheodyne® 7125 injector port. UV spectra were recorded on a Hewlett Peckard® 8452A diode array spectrophotometer (France). IR spectra were recorded on a Jasco® IR-810 infrared spectrophotometer (Japan). Mass spectra both low and high resolutions were operated on an MAT 95 XL mass spectrometer (Germany). NMR spectra were recorded on an FT-NMR, Varian Unity® Inova 500 spectrometer (Germany) at 500 MHz for ^{1}H.

2.2 Isolation and purification of marine gliding bacteria

Modified SWG medium (*L*-glutamic acid monosodium salt, 1 g; NH_4NO_3, 0.01 g; K_2HPO_4, 0.01 g; agar 15 g; seawater, 1 L) (Hosoya *et al*., 2006) and modified SWG medium with 0.1% (w/v) crystal violet (Zhang *et al*., 2003) were used for the isolation of marine gliding bacteria. Crystal violet was used in order to inhibit the fast growing Gram-positive bacteria. The marine specimens such as algae, biofilms, and invertebrate and plant materials were collected from various locations along the coastlines of Thailand by snorkeling and scuba diving. Specimens, after rinsing with sterile seawater, were cut into small pieces by a sterilized sharp blade and placed on the isolation media in Petri dishes before incubating at room temperature. Daily examination for swarm colony and fruiting body formation was performed under a stereomicroscope. A clean edge of swarm colony was cut into a small agar pieces using a sterilized sharp blade and transferred to a fresh modified SAP2 agar plate (tryptone, 1 g; yeast extract, 1 g; agar, 15 g; seawater, 1 L) (Reichenbach, 1991). The sub-cultured plate was incubated at room temperature until the swarm colony of gliding bacteria could be observed again. Repeated subculturing or single cell isolation by a micromanipulator was used as the purification methods until the pure culture of gliding bacterium could be obtained (Li *et al*., 2002; Iizuka *et al*., 1998; Skerman, 1968 cited in Sly and Arunpairojana, 1987).

2.3 Preservation of marine gliding bacterial isolates

The 3 day-old cultures of marine gliding bacteria were harvested and suspended in 10% skimmed milk. The isolates were then preserved by liophilization or freeze-drying method. The identified isolates obtained from this study were deposited at the culture collection of Microbiological Resources Center (Bangkok-MIRCEN), the Thailand Institute of Scientific and Technological Research (TISTR).

2.4 Identification of marine gliding bacterial isolates by 16S rRNA gene sequence analysis

2.4.1 Preparation of marine gliding bacterial DNA

The genomic DNA of each isolates was extracted using a Wizard® genomic DNA purification kit (Promega) according to the manufacturer's instruction. In brief, cell suspension in liquid SAP2 medium was centrifuged at 8,000 rpm to obtain cell pellets. Pellets in a microtube were re-suspended in 480 µl of 50 mM EDTA and 120 µl of lytic enzyme was added and gently mixed by pipetting. After incubation at 37 °C for 60 min, mixture was centrifuged at 14,000 ×g for 3 min. Supernatant was removed and remaining cells were lysed with 600 µl of nuclei lysis solution, mixed by repeated pipetting. After incubation at 80°C for 5 min, sample was cooled to room temperature and 3 microliters of RNase solution were added to a microtube before mixing and incubating at 37°C for 60 min. Protein in lysed cells was removed by precipitation with an addition of 200 µl of protein precipitation solution and incubating in ice bath for 5 min. After centrifugation at 14,000 ×g for 3 min, the supernatant was transferred to a clean tube containing 600 µl of isopropanol, for DNA precipitation, and the mixture was mixed and centrifuged at 14,000 ×g for 2 min. The supernatant was discarded and the crude genomic DNA was rinsed with 600 µl of 70% ethanol and centrifuged at 14,000 ×g for 2 min. The crude genomic DNA pellet was then dried at room temperature for 15 min before rehydrating in 100 µl of rehydration solution at 65°C for 1 h. Crude genomic was analysed for purity by an agarose gel electrophoresis (0.8 % agarose in TAE buffer) and compared with DNA markers. The crude genomic DNA was stored at -20°C until further use.

2.4.2 Amplification of 16S rRNA fragment

Each of 16S rRNA fragment from crude genomic DNA of marine gliding bacteria was amplified by a PCR machine (GeneAmp® PCR System 9700, Applied Biosystem, Foster City, CA, USA and GeneAmp PCR System 2400, PERKIN ELMER, Norwalk CT, USA) with the universal primer sets of BF1 (5'-GAGTTTGATCATGGCTCAG-3') and BR1 (5'-CGGTTACCTTGTTACGACTT-3'). The PCR reaction mixture consisted of 30 to 50 ng of

genomic DNA, 5 µl of 10x*ExTaq*™ buffer, 4 µl of 2.5 mmol deoxynucleoside triphosphate mixtures, 1 µl of 20 µmol of each primer and 0.25 µl of 5U of *ExTaq*™ polymerase. The PCR condition included an initial denaturation at 94°C for 2 min; 25 cycles of amplification at 94°C for 10 s, 55°C for 30 s, and 68 °C for 2 min and finally an extension at 72°C for 10 min**.** The amplified products were examined by an agarose gel electrophoresis (1% agarose in TAE buffer) compared with standard DNA markers. The amplified RNA was stored at -20°C until further use.

2.4.3 Purification of amplified 16S rRNA product

Amplified product was purified by GFX PCR DNA and gel band purification kit (Amersham Biosciences, Buckinghamshire, England) in order to remove primers and free nucleotides. Purification procedure was done according to the manufacturer's instruction. GFX column was placed into a collection tube in which 500 µl of capture buffer was added. PCR product was transferred to GFX column and mixed by pipetting. After centrifugation at 14,000 ×g for 30 sec, the solution in collection tube was discarded. GFX column was back placed inside the collection tube and 500 µl of wash buffer was added before centrifugation at 14,000 ×g for 30 sec. GFX column was transferred to a fresh 1.5 ml microcentrifuge tube and 50 µl of elution buffer (TE pH 8.0) was applied. The sample was incubated at room temperature for 1 min and the purified 16S rRNA fragment was obtained in 1.5 ml microcentrifuge tube after centrifugation at 14,000 ×g for 1 min. The purified 16S rRNA fragment was checked for purity by an agarose gel electrophoresis (1% agarose in TAE buffer) that compared with DNA marker. The purified RNA was stored at -20°C until further use.

2.4.4 Sequencing of 16S rRNA fragment

The PCR product was directly sequenced by the ABI PRISM™ BigDye terminator cycle sequencing ready reaction kit version 3.1 (Applied Biosystem, Foster City, CA, USA). The internal oligonucleotide primers used for full length sequencing of amplified 16S rRNA fragment were BF1 (5'-GAGTTTGATCATGGCTCAG-3') and BR1 (5'-CGGTTACCTTGTTACGACTT-3'), 339F (5'-CTCCTACGGGAGGCAGCAG-3'), 785F (5'-GGATTAGATACCCTGGTAGTC-3'), 1099F (5'-GCAACGAGCGCAACCC-3'), 536R (5'-GTATTACCGCGGCTGCTG-3'), 802R (5'-TACCAGGGTATCTAATCC-3') and 1115R (5'-AGGGTTGCGCTCGTTG-3') (Nakagawa *et al*., 2001). Each sequencing reaction mixture consisted of 1.5 µl of BigDye terminator, 1 µl of 5xBigDye buffer, 4.5 µl of MilliQ water, 1 µl of 2 µmol of specific primer and 2 µl of DNA template (purified PCR product). Then DNA was precipitated by adding forty microliters of ethanol/sodium acetate solution (the mixture of 3.0 µl of 3 M sodium acetate pH 4.6, 14.5 µl of deionized water and 62.5 µl of non-denatured 95-99% ethanol) into 1.5 microcentrifuge tube containing 10 µl of sequencing reaction mixture and mixed by pipetting. After incubation at room temperature for 15 min, the sample was centrifuged at 14,500 ×g for 20 min and supernatant was carefully aspirated by pipetting. The sample was centrifuged at 14,500 ×g for 5 min after adding of 250 µl of 70% ethanol. After supernatant aspiration, the tube was dried at 60°C for 5 min in heat box to obtain precipitated RNA. The pellet was resuspended with 20 µl of TSR (Template Suppresion Reagent) solution. After mixing, the sample was heated at 95°C for 2 min and chilled on ice both until loading to an ABI PRISM™ 310 Genetic Analyzer (Applied Biosystem, Foster City, CA, USA) with 50°C during operation.

2.4.5 Phylogenetic analysis

Nucleotide sequences of all marine gliding bacterial isolates were analyzed and edited manually or by using BioEdit software (Hall, 1999). The 16S rRNA gene sequences were compared with sequences available in GenBank/EMBL/DDBJ databases using the BLASTN program to search for close evolutionary relatives. Alignments of the 16S rRNA gene sequences of the isolates and phylogenetically related genera were carried out by using the CLUSTAL X program version 1.83 (Thompson *et al*., 1997). Nucleotide substitution rates (K_{nuc} values) were determined (Kimura, 1980). Distance matrix trees were constructed by using three algorithms including the neighbor-joining (Saitou and Nei, 1987), maximum parsimony and maximum likelihood method. Alignment gaps and unidentified base positions were not taken into consideration in the calculations. The topology of the phylogenetic tree was evaluated by performing a bootstrap analysis with 1000 trials, except maximum likelihood with 100 trials.

2.5 Cultivation of marine gliding bacteria for production of crude extracts

Forty-two isolates of marine gliding bacteria were selected from culture collection and were cultivated in four modified cultivation media including Vy/2 medium [baker's yeast paste, 5 g; seawater, 1 L] (Iizuka *et al*., 2003); RL 1 medium [peptone, 3 g; yeast extract, 2 g; KNO_3, 0.5 g; vitamin B_{12}, 0.5 μg/L; seawater, 1 L] (Spyere *et al*., 2003); CY medium [casitone, 10g; malt extract, 2g; yeast extract, 1g; seawater, 1 L] (Suzuki *et al*., 1998) and SK medium [skim milk, 5 g; yeast extract, 3 g; $MgSO_4$, 1 g; seawater, 1 L]. At first, each isolate was grown on SAP2 agar medium and cut into small pieces before transferring into 250-ml Erlenmeyer flasks containing 30 ml of SAP2 broth medium. The inoculating flask was incubated and shaken at 25 °C, 200 rpm for 48-72 h. Five milliliters of inoculum was transferred into each of the four cultivation flasks (100 ml in 250-ml Erlenmeyer flasks) containing 2 grams of amberlite XAD-16 resin. Amberlite XAD-16 resin is polymeric adsorbent which added to adsorb hydrophobic molecules from polar solvent (cultivation media). All cultivating flasks were incubated and shaken at 25 °C, 200 rpm for 7 days which that was the stationary phase.

2.6 Crude extract preparation

After cultivation was completed, the amberlite XAD-16 resins were collected by filtering through a nylon mesh and washed with 100-200 ml of deionized water in order to remove salt and remaining culture medium. The resins were left to dry at room temperature for 10 min and then soaked twice for 3 hr with 100 ml of methanol. Crude extract was obtained after combining the methanol fractions and evaporating by a rotary evaporator.

2.7 Cytotoxic assay

Crude extracts were screened for cytotoxicity by measuring the percentage of inhibition of the cancer cells whereas the cytotoxicity of a pure compound was latetr measured as an IC_{50}. Four human cell lines including MCF-7 (breast adenocarcinoma), HT-29 (colon cancer), HeLa (cervical cancer) and KB (oral cancer) were used for the screening of cytotoxic activity by a Sulphorodamine B (SRB) assay (Skehan *et al*., 1990). In brief, monolayer cultures of each cell line in 96-well microtiter plate (approximately, $2x10^3$cells/well) were treated with 25 µg/ml of each sample produced from marine gliding bacteria. The plate was incubated in a CO_2 incubator according to the reported conditions (5% CO_2, 95% humidity, 37 °C) for 7 days, at the midway of cultivation the medium was refreshed once (~72 h). After incubation, survived cells were fixed with ice-cold 40% trichloroacetic acid and then washed with distilled water, air-dried and stained with 0.4% Sulphorodamine B (SRB) in 1% acetic acid. Residual SRB was washed with 1% acetic acid and air-dried. The stained dye on the fixed cells was solubilized with 10 mM Tris base for 20 min on a gyratory shaker. As internal control of this experiment, one column of 96-well microtiter plate containing $2.0x10^3$cells/well of each cancer cell line was not treated with crude extract. Percentage of inhibition was calculated from survival rate as described below.

$$\% \text{ inhibition} = [(OD_{492}\,\text{Control} - OD_{492}\,\text{Sample}) / (OD_{492}\,\text{Control})] \times 100$$

For primary cytotoxic screening, a threshold of 80% cell-growth inhibition was used as a cut-off for positive cytoxicity.

The IC_{50} value (the concentration causing 50% growth inhibition) of the pure compound was determined by ploting a dose-response curve between the compound concentration and percent growth inhibition. In this study, camptothecin was used as the positive control.

2.8 Large-scale fermentation and crude extract preparation

Large scale fermentation of selected isolates showing strong cytotoxicity began with an inoculum preparation step in 6× Erlenmeyer flasks (250 ml) containing 30 ml of SAP2 medium, incubated at 25 °C, 200 rpm for 72 h. Five milliliters of inoculum were added into 250-ml Erlenmeyer flasks (×80) containing 100 ml of production medium, depending on the previous screening result, and two grams of amberlite XAD-16 resins. All flasks were incubated at the same conditions of pre-culture step for 7 days. At the end of the fermentation, the resins were separated from broth by filtration with a nylon mesh. After rinsing with deionized water and drying at room temperature, the resins were soaked twice in 100 ml of methanol. Crude extract was obtained after combining methanol fraction and evaporation with a rotary evaporator.

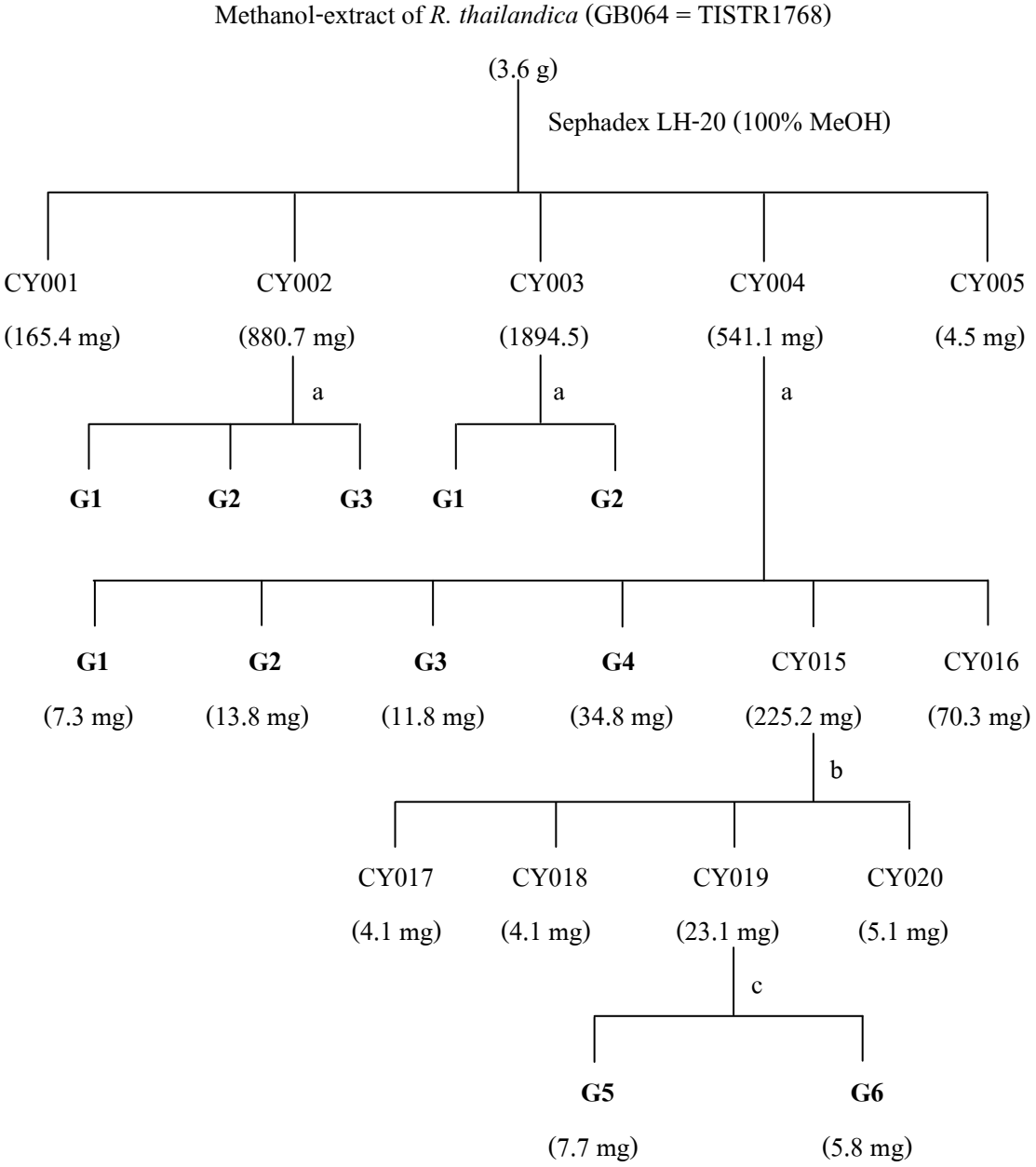
2.9 Isolation and structure elucidation of cytotoxic compounds

2.9.1 Compounds G1-G4

Crude extract (3.6 g), which was aliquoted from 8.8 g obtained from 8 liters fermentation of marine gliding bacterium *Rapidithrix thailandica* strain GB064 (TISTR1768) in modified CY medium, was subjected to a size exclusion gel column chromatography on Sephadex LH-20 using methanol as an eluent yielding five fractions (CY001-CY005). The bioassay guided fractionation of CY004 (541.1 mg) was further separated and purified by a preparative HPLC (Luna 100 reverse-phase C-18, 250×10 mm, 10 µm, Phenomenex) using 30:70 methanol in water as a mobile phase with a flow rate of 4.5 ml/min; detection: UV detection at 210 nm yielding compounds **G1** (7.3 mg), **G2** (13.8 mg), **G3** (11.8 mg) and **G4** (34.8 mg) at retention times of 20, 23, 28 and 31 minutes, respectively (Scheme 1).

2.9.2 Compounds G5 and G6

The fraction CY015 (255.2 mg) obtained from 2.9.1 was further separated by preparative HPLC (Luna 100 reverse-phase C-18, 250×10 mm, 10 µm, Phenomenex) using 15:85 methanol in water as a mobile phase with a flow rate of 4.5 ml/min; detection: UV detection at 210 nm yielding four fractions (CY017-CY020). Fraction CY019 (23.1 mg) was further subjected to a gel column chromatography on Silica gel-60 using methanol in dichloromethane (5:95) as mobile phase yielding compounds **G5** (7.7 mg) and **G6** (5.8 mg). The isolation scheme of **G5** and **G6** is shown in Scheme 1.



Note: a; RP C-18 HPLC, 30:70 MeOH in H_2O

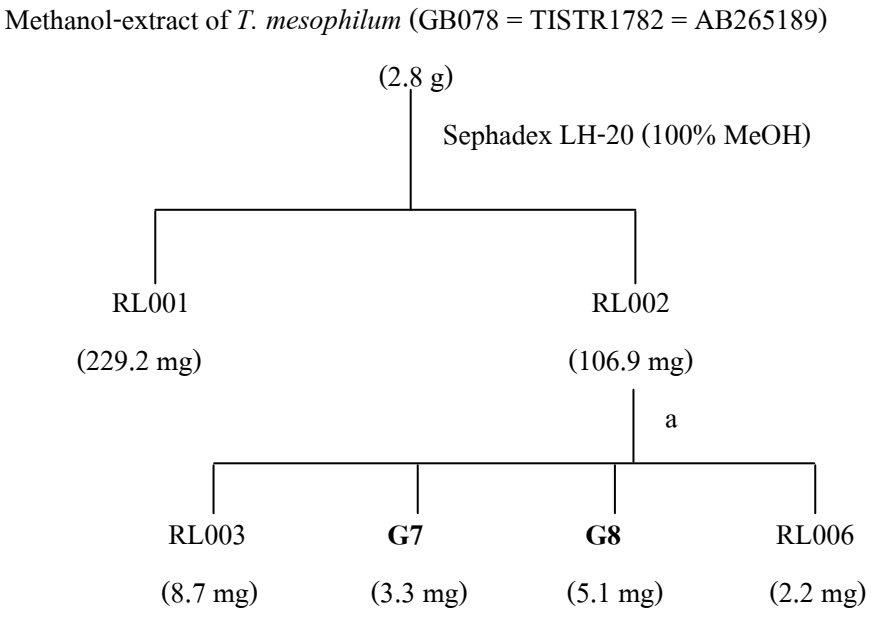
b; RP C-18 HPLC, 15:85 MeOH in H_2O

c; Silicagel-60, 5:95 MeOH in DCM

Scheme 1. Isolation scheme for compounds **G1-G6**

2.9.3 Compounds G7 and G8

Crude extract (2.8 g), which was aliquoted from 4.6 g obtained from 8 liters fermentation of marine gliding bacterium *Tenacibaculum mesophilum* strain GB078 (TISTR1782 = AB265189) in modified RL1 medium, was subjected to a size exclusion gel column chromatography on Sephadex LH-20 using methanol as an eluent yielding two fractions (RL001 and RL002). Fraction RL002 (106.9 mg) was further separated and purified by a preparative HPLC (Luna 100 reverse-phase C-18, 250×10 mm, 10 µm, Phenomenex) using 30:70 methanol in water as a mobile phase with a flow rate of 3.5 ml/min; detection: UV detection at 210 nm yielding compounds **G7** (3.3 mg) and **G8** (2.2 mg) at retention times of 10 and 13 minutes, respectively (Scheme 2).

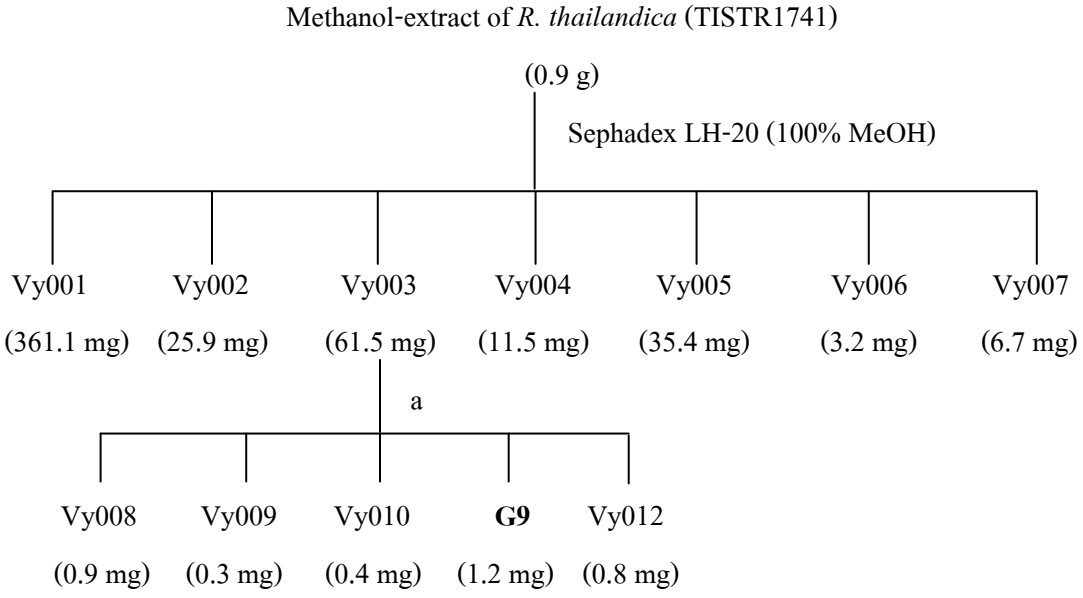


Note: a; RP C-18 HPLC, 30:70 MeOH in H_2O

Scheme 2. Isolation scheme of compounds **G7** and **G8**

2.9.4 Compound G9

Crude extract (0.9 g), obtained from 4 liters fermentation of marine gliding bacterium *Rapidithrix thailandica* strain TISTR1741 (AB265183) in modified Vy/2 medium, was subjected to a size exclusion gel column chromatography on Sephadex LH-20 using methanol as an eluent yielding seven fractions (Vy001-Vy007). The cytotoxic fraction, Vy003 (61.5 mg), was further separated and purified by a preparative HPLC (Luna 100 reverse-phase C-18, 250×10 mm, 10 μm, Phenomenex) using 30:70 methanol in water as a mobile phase with a flow rate of 4.5 ml/min; detection: UV detection at 210 nm yielding compounds **G9** (1.2 mg) at retention times of 15 minutes, respectively (Scheme 3).



Note: a; RP C-18 HPLC, 30:70 MeOH in H_2O

Scheme 3. Isolation scheme of compound **G9**

2.10 Physico-chemical properties of isolated compounds

Cyclo(L-Pro-D-Phe) [G1]: Brownish orange glass: $[\alpha]_D$ +95.7 (*c* 0.06; MeOH); UV (MeOH) λ_{max} (log ε) 210 (3.80) nm; IR (thin film) ν_{max} 3250, 2925, 1660, 1450 cm^{-1}; ^1H and ^{13}C NMR (500 MHz for ^1H; DMSO-d_6) see Table 7; EIMS *m/z* (% relative intensity) 244 $[M]^+$ (49), 153 (35) , 125 (100), 91 (39) (calcd for $C_{14}H_{16}N_2O_2$).

Cyclo(L-Pro-L-Phe) [G2]: Brownish orange glass: $[\alpha]_D$ -88.9 (*c* 0.25; MeOH); UV (MeOH) λ_{max} (log ε) 208 (3.92) nm; IR (thin film) ν_{max} 3250, 2925, 1660, 1450 cm^{-1}; ^1H and ^{13}C NMR (500 MHz for ^1H; DMSO-d_6) see Table 7; EIMS *m/z* (% relative intensity) 244 $[M]^+$ (91), 153 (31), 125 (100), 91 (39) (calcd for $C_{14}H_{16}N_2O_2$).

***N*-(2-phenylethyl) acetamide [G3]:** Brownish orange glass: $[\alpha]_D$ -70.2 (*c* 0.09; MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.12) nm; IR (thin film) ν_{max} 3280, 2925, 1650, 1450 cm^{-1}; ^1H and ^{13}C NMR (500 MHz for ^1H; $CDCl_3$+CD_3OD) see Table 8; EIMS *m/z* (% relative intensity) 163 $[M]^+$ (26), 104 (100), 91 (25), 77 (9); HREIMS *m/z* 163.0958 (calcd for $C_{10}H_{13}NO$, 163.0997).

3-(2'-aminophenyl)-pyrrole [G4]: Brownish orange glass: $[\alpha]_D$ -65.6 (*c* 0.09; MeOH); UV (MeOH) λ_{max} (log ε) 212 (4.07), 302 (3.34) nm; IR (thin film) ν_{max} 3400, 2925, 1610, 1500, 1450 cm^1; ^1H and ^{13}C NMR (500 MHz for ^1H; $CDCl_3$) see Table 9; EIMS *m/z* (% relative intensity) 158 $[M]^+$ (100), 130 (73); HREIMS *m/z* 158.0852 (calcd for $C_{10}H_{10}N_2$, 158.0842).

Cyclo(L-Pro-D-Tyr) [G5]: Brownish orange glass: $[\alpha]_D$ -91.7 (*c* 0.09; MeOH); UV (MeOH) λ_{max} (log ε) 208 (4.08), 224 (4.04), 278 (3.27) nm; IR (thin film) ν_{max} 3250, 2930, 1660, 1450 cm^{-1}; ^1H and ^{13}C NMR (500 MHz for ^1H; $CDCl_3$) see Table 10; EIMS *m/z* (% relative intensity) 260 $[M]^+$ (27), 154 (100), 107 (95), 70 (28) (calcd for $C_{14}H_{16}N_2O_3$).

Cyclo(L-Pro-L-Tyr) [G6]: Brownish orange glass: $[\alpha]_D$ +22.4 (*c* 0.23; MeOH); UV (MeOH) λ_{max} (log ε) 208 (3.89), 226 (3.81), 276 (3.08) nm; IR (thin film) ν_{max} 3250, 2930, 1660, 1450 cm^{-1}; ^1H and ^{13}C NMR (500 MHz for ^1H; DMSO-d_6) see Table 10.

Cyclo(8-hydroxy-L-Pro-L-Leu) [G7]: Brownish orange glass: $[\alpha]_D$ -23.1 (*c* 0.06; MeOH); UV (MeOH) λ_{max} (log ε) 208 (4.13) nm; IR (thin film) ν_{max} 3250, 2925, 1650, 1450 cm^{-1}; ^1H and ^{13}C NMR (500 MHz for ^1H; $CDCl_3$+CD_3OD) see Table 11; ESIMS *m/z* (% relative intensity) 249 $[M+Na]^+$ (18) (calcd for $C_{11}H_{18}N_2O_3$).

Cyclo(8-hydroxy-L-Pro-D-Phe) [G8]: Brownish orange glass: $[\alpha]_D$ -76.7 (*c* 0.06; MeOH); UV (MeOH) λ_{max} (log ε) 210 (4.13) nm; IR (thin film) ν_{max} 3250, 2925, 1650, 1450 cm^{-1}; ^1H and ^{13}C NMR (500 MHz for ^1H; $CDCl_3$+CD_3OD) see Table 12; ESIMS *m/z* (% relative intensity) 283 $[M+Na]^+$ (100) (calcd for $C_{14}H_{16}N_2O_3$).

3-(2-Amino-phenyl)-5-methoxy-1,5-dihydro-pyrrol-2-one [G9]: Brownish orange glass: $[\alpha]_D$ +11.4 (*c* 0.2; MeOH); UV (MeOH) λ_{max} (log ε) 208 (3.23), 230 (3.10) nm; IR (thin film) ν_{max} 3300, 2925, 1700, 1620, 1450 cm^{-1}; ^1H and ^{13}C NMR (500 MHz for ^1H; $CDCl_3$) see Table 13; EIMS *m/z* (% relative intensity) 205 $[M+H]^+$ (89), 173 (14); HREIMS *m/z* 205.0970 $[M+H]^+$ (calcd for $C_{11}H_{12}O_2N_2$, 205.0977).

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Isolation and purification of marine gliding bacteria

In this study, twenty-seven marine gliding bacteria were isolated from the marine specimens collected from three locations in the Gulf of Thailand including Amphur Sattahip, Chonburi province; Tao Island, Suratthani province and Kra Island, Nakhon Si Thammarat province. The specimens including biofilm, algae, invertebrates and plant debris were collected either from the seafloor (dept, 5-20 m), by scuba diving, or in the inter-tidal zone as shown in Table 3.

Samples obtained from underwater in Amphur Sattahip, Chonburi province yielded the highest number of isolates (18 strains) followed by the samples collected from Kra Island, Nakhon Si Thammarat province (9 isolates). Although we were able to isolate 8 gliding bacteria from Tao Island, Suratthani province, all of the isolates were unable to survive throughout the course of isolation and identification. All 27 isolates were obtained from five different types of specimens including biofilm, sponges, algae, plant and animal materials. The result also showed that biofilm was the richest source of marine gliding bacteria when compared to other specimens (Fig. 14). This may be due to the fact that marine biofilm is the consortia of microorganisms in the ocean. Therefore, gliding bacteria which require surface for attaching and gliding as well as nutrients from other microorganisms are more abundant than in other specimens. Sponges which are filter feeders that host several groups of microorganisms also tend to be the potential sources of gliding bacteria similar to biofilm. Several studies on microbial diversity in sponges also provided the strong supports that marine sponges could harbour both culturable and unculturable microorganisms i.e. *Aerothermobacter marianis*, *Ferromicrobium acidophilum*, *Bathymodiolus thermophilus*, *Riftia pachyptilla*, *Anodontia phillipiona*, and *Cytophaga* sp. in *Rhopaloeides odorabile* (Webster *et al*., 2001); *Actinoalloteichus*, *Micromonospora*, *Nocardia*, *Nocardiopsis*, *Pseudonocardia*, *Rhodococcus*, and *Streptomyces* in *Hymeniacidon perleve* (Zhang *et al*., 2006). The microorganisms associated with sponges are known as ‘symbiont’ and these in some cases

suggesting possible symbiotic functions (Webster *et al*., 2001). The biology of the bacterium-sponge relationship has elicited considerable interests among researchers investigating marine organisms as the sources of natural products. Antimicrobial compounds have been isolated from sponge-associated bacteria on numerous occasions. This has prompted the suggestion that microbial symbionts play a role in the defense of their host sponge (Webster *et al*., 2001). However, there has been no specific report on isolation of gliding bacteria from marine sponge as well as their secondary metabolites. This study is, therefore, the first report on the isolation of marine gliding bacteria from sponges together with the study of their metabolites. According to isolation results, it was also found that algae were the potential sources of gliding bacteria eventhough the total numbers of the isolates were less than what were obtained from biofilm. This might be due to the fact that the numbers of algae collected in this study were much smaller than numbers of biofilm and sponge samples. Previous studies by other researchers also reported that algae could be potential sources of gliding bacteria which are epiphytes (Barbeyron *et al*., 2001; Johansen *et al*., 1999). These epibionts may play a protective role by releasing chemicals into the surrounding seawater that help prevent extensive fouling of the algal surface. Interestingly, some of gliding bacterial isolates showed very high agarolytic activity suggesting their ability to attach algae and obtain nutrients for growth. Johansen *et al*. (1999) suggested that gliding bacteria are involved in an active decomposition of the alga, where the bacteria use the macroalga not only as a substratum but also as a substrate. Members of the CFB group therefore play a key role in the biodegradation of macroalgae in seawater and carbon cycling in the marine food chain.

In case of the purification of isolates, subculturing and micromanipulation were used successfully to obtaine marine gliding bacteria in this study. All of isolates (18 isolates) obtained from Amphur Sattahip were purified by a subculturing technique. However, this purification method is very time consuming and the isolation of a single cell by a micromanipulator was later employed in order to isolate gliding bacteria from specimens collected from Kra Island after the swarm colony could be detected on the isolation plate. Micromanipulation technique was much less time consuming and more efficient although special skill in isolation of the single cell was required. Although Zhang and colleagues (2003) believed that micromanipulator was a better solution, they could not obtain satisfied results which might be the result of lacking in experience.

When media compositions were compared, the modified SWG medium seemed to be the most suitable medium for the isolation of marine gliding bacteria because of its low nutrient (*L*-glutamic acid monosodium salt, 1 g; NH_4NO_3, 0.01 g; K_2HPO_4, 0.01 g; agar 15 g; seawater, 1 L). Minimal medium is beneficial for the isolation of gliding bacteria because it can minimize the overgrowth of other competitive or fastidious microorganisms. Moreover, the addition of 0.1% crystal violet proved to further inhibit Gram-positive bacterial contaminant during the isolation as previously reported (Zhang *et al*., 2003). Once the pure cultures were obtained, they were transferred for maintaining as working for further study.

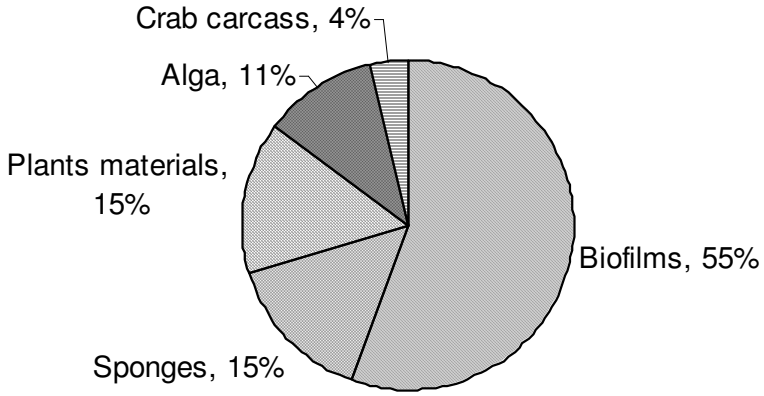


Figure 14. Percentages of marine gliding bacteria isolated from five kinds of marine specimens

Table 3. Summary of marine gliding bacteria isolated in Thailand

No.	Code	TISTR Accession No.	Source	Location	Purification technique
1	GB057	TISTR1760	Wood material	Satthahip, Chonburi	Subculturing
2	GB058	TISTR1761	Brown alga	Satthahip, Chonburi	Subculturing
3	GB059	TISTR1762	Red alga	Satthahip, Chonburi	Subculturing
4	GB060	TISTR1763	Biofilm	Satthahip, Chonburi	Subculturing
5	GB061	TISTR1764	Biofilm	Satthahip, Chonburi	Subculturing
6	GB062	TISTR1766	Biofilm	Satthahip, Chonburi	Subculturing
7	GB063	TISTR1767	Biofilm	Satthahip, Chonburi	Subculturing
8	GB064	TISTR1768	Blue-green alga	Satthahip, Chonburi	Subculturing
9	GB065	TISTR1769	Biofilm	Satthahip, Chonburi	Subculturing
10	GB066	TISTR1770	Biofilm	Satthahip, Chonburi	Subculturing
11	GB067	TISTR1771	Biofilm	Satthahip, Chonburi	Subculturing
12	GB068	TISTR1772	Biofilm	Satthahip, Chonburi	Subculturing
13	GB069	TISTR1773	Biofilm	Satthahip, Chonburi	Subculturing
14	GB070	TISTR1774	Biofilm	Satthahip, Chonburi	Subculturing
15	GB071	TISTR1775	Biofilm	Satthahip, Chonburi	Subculturing
16	GB072	TISTR1776	Biofilm	Satthahip, Chonburi	Subculturing
17	GB073	TISTR1777	Sponge	Satthahip, Chonburi	Subculturing
18	GB084	TISTR1765	Biofilm	Satthahip, Chonburi	Subculturing
19	GB074	TISTR1778	Sponge	Kra Island, Nakhon Si Thammarat	Micromanipulation
20	GB075	TISTR1779	Decaying leaf	Kra Island, Nakhon Si Thammarat	Micromanipulation
21	GB076	TISTR1780	Decaying leaf	Kra Island, Nakhon Si Thammarat	Micromanipulation
22	GB077	TISTR1781	Decaying leaf	Kra Island, Nakhon Si Thammarat	Micromanipulation
23	GB078	TISTR1782	Sponge	Kra Island, Nakhon Si Thammarat	Micromanipulation
24	GB079	TISTR1783	Sponge	Kra Island, Nakhon Si Thammarat	Micromanipulation
25	GB080	TISTR1784	Biofilm	Kra Island, Nakhon Si Thammarat	Micromanipulation
26	GB081	TISTR1785	Crab carcass	Kra Island, Nakhon Si Thammarat	Micromanipulation
27	GB082	TISTR1786	Biofilm	Kra Island, Nakhon Si Thammarat	Micromanipulation

3.2 Identification and classification of marine gliding bacterial isolates by 16S rRNA gene sequence analysis

In this study, eighty-four marine gliding bacteria, comprising of 27 isolates obtained from Amphur Sattahip, Chonburi province and Kra Island, Nakhon Si Thammarat province and 57 isolates obtained from the Andaman Sea (including Trang, Krabi and Phuket provinces) which were previously deposited at the culture collection of Microbiological Resource Centre (Bangkok-MIRCEN), Thailand Institute of Scientific and Technological Research (TISTR). All isolates were subjected to the identification by 16S rRNA gene sequence analysis. Generally, all isolates were characterized as Gram-negative bacteria with swarm colony and gliding motility. The 16S rRNA gene sequences of selected isolates were analyzed and aligned with 16S rRNA gene sequences in GenBank/EMBL/DDBJ databases and the results are shown in Table 4. Base on sequence similarity, it clearly indicates that marine gliding bacteria isolated in Thailand related to six different groups in the major group of *Cytophaga-Flavobacterium-Bacteroides* (CFB) including *Flavobacteriaceae bacterium* (with 90% similarities), *Tenacibaculum mesophilum* (with 99%-100% similarities), *Aureispira maritima* (with 99% similarities), *Aureispira marina* (with 99% similarities), *Rapidithrix thailandica* (with 97%-99% similarities), and *Fulvivirga kasyanovii* (with 98% similarities). The further analyses using almost complete 16S rRNA gene sequences of all twenty-seven representative strains and selected neighbors-species in CFB group from GenBank/EMBL/DDBJ databases for constructing the phylogenetic tree was done by the CLUSTAL X version 1.83 (Thompson *et al*., 1997).

The phylogenetic trees from three algorithms including neighbor-joining, maximum parsimony and maximum likelihood (Fig. 15, 16 and 17) are shown same informations. The results indicated that these isolates could devide into six groups and clearly demonstrated that the diversity of marine gliding bacteria isolated in Thailand is uniquely rich. As the members of group 1 (GB058 = TISTR1761 and GB060 = TISTR1763) formed a distinct lineage separating from the neighbor species, *Flavobacteriaceae bacterium* (DQ423479) and the 16S rRNA gene sequence similarities between group 1 and this neighbor species was only 90%, it was sufficient to propose a novel genus (Iizuka *et al*., 2003; Nedashkovskaya *et al*., 2003; Barbeyron *et al*., 2001; Johansen *et al*., 1999). Scanning electron micrograph of GB060 (TISTR1763) showed the cell morphology with filamentous shape (Fig. 18).

Table 4. 16S rRNA gene sequence analysis results of representative isolates

No.	Strain	Closely related species	% Similarity
1	GB058 (TISTR1761)	*Flavobacteriaceae bacterium*	90% (1319/1458)
2	GB060 (TISTR1763)	*Flavobacteriaceae bacterium*	90% (1318/1455)
3	GB019 (TISTR1755 = AB265190)	*Tenacibaculum mesophilum*	99% (1443/1446)
4	GB074 (TISTR1778 = AB265188)	*Tenacibaculum mesophilum*	99% (1442/1446)
5	GB078 (TISTR1782 = AB265189)	*Tenacibaculum mesophilum*	100% (1444/1444)
6	GB080 (TISTR 1784)	*Tenacibaculum mesophilum*	99% (1419/1421)
7	GB033 (TISTR1692)	*Aureispira maritima*	99% (1392/1393)
8	TISTR1726 (AB278130)	*Aureispira maritima*	100% (1432/1432)
9	GB055 (AB265191)	*Aureispira maritima*	99% (1413/1416)
10	GB007 (TISTR1721)	*Aureispira marina*	99% (1399/1404)
11	GB051 (TISTR1719 = AB245933)	*Aureispira marina*	100% (1446/1446)
12	GB043 (TISTR1728 = AB245934)	*Aureispira marina*	99% (1442/1445)
13	GB052 (TISTR1731 = AB245935)	*Aureispira marina*	99% (1442/1444)
14	GB002 (TISTR1741 = AB265183)	*Rapidithrix thailandica*	99% (1065/1067)
15	GB009 (TISTR1742)	*Rapidithrix thailandica*	99% (1064/1067)
16	GB003 (TISTR1747 = AB265184)	*Rapidithrix thailandica*	99% (1065/1068)
17	GB016 (TISTR1749 = AB265185)	*Rapidithrix thailandica*	99% (1065/1068)
18	GB015 (TISTR1750 = AB265192)	*Rapidithrix thailandica*	100% (1465/1465)
19	GB022 (TISTR1710)	*Rapidithrix thailandica*	99% (1065/1067)
20	GB057 (TISTR1760 = AB265186)	*Rapidithrix thailandica*	99% (1062/1066)
21	GB062 (TISTR1766)	*Rapidithrix thailandica*	99% (1065/1067)
22	GB064 (TISTR1768)	*Rapidithrix thailandica*	99% (1063/1067)
23	GB068 (TISTR1772 = AB265187)	*Rapidithrix thailandica*	99% (1061/1064)
24	GB084 (TISTR1765)	*Rapidithrix thailandica*	99% (1064/1067)
25	GB005 (TISTR1736 = AB284517)	*Rapidithrix thailandica*	97% (1019/1041)
26	GB076 (TISTR1780)	*Fulvivirga kasyanovii*	98% (1332/1346)
27	GB077 (TISTR1781)	*Fulvivirga kasyanovii*	98% (1329/1343)

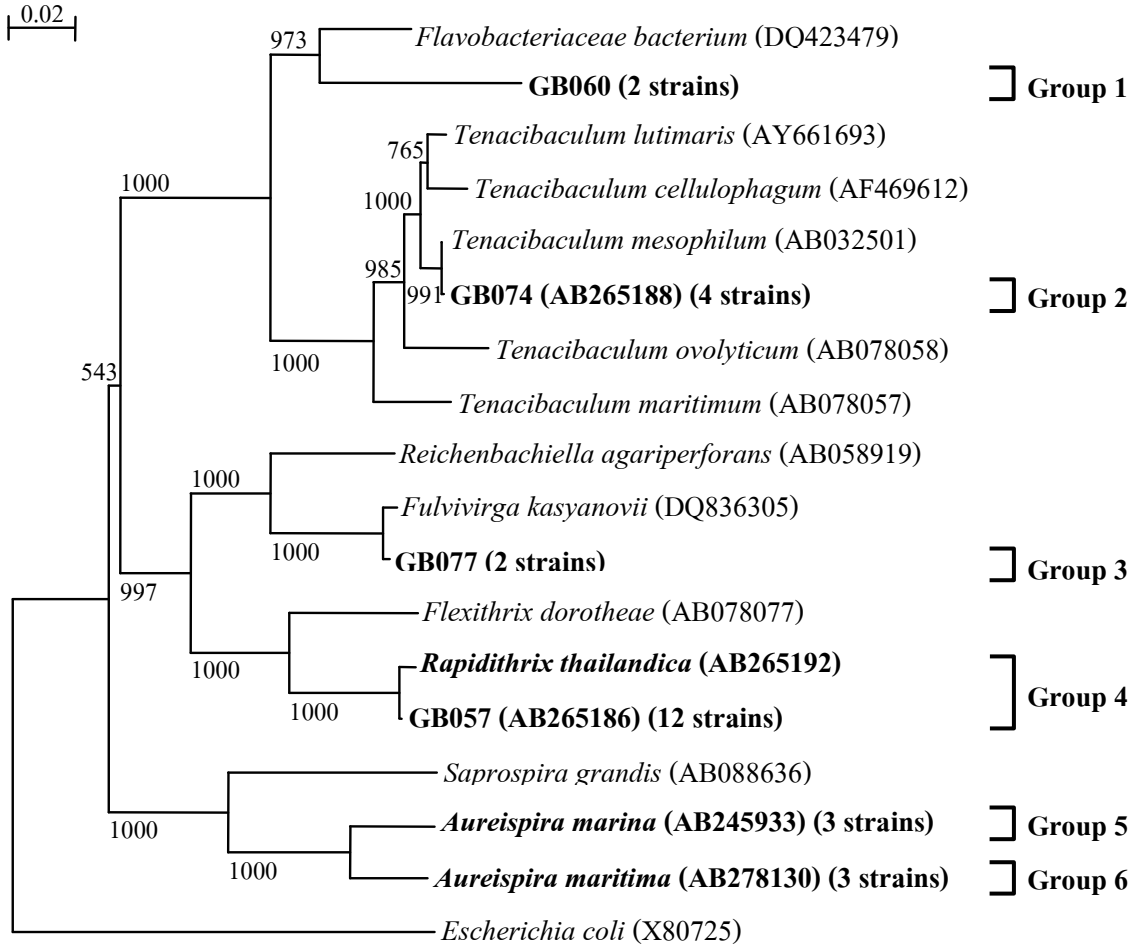


Figure 15. Neighbor-joining phylogenetic tree of marine gliding bacterial isolates and related members of the CFB group based on 16S rRNA gene sequence analysis using CLASTAL X version 1.83. Numbers within the phylogenetic tree indicate the percentages of occurrence of the branching order in 1000 bootstrapped trees. Bar = 0.02 K_{nuc}

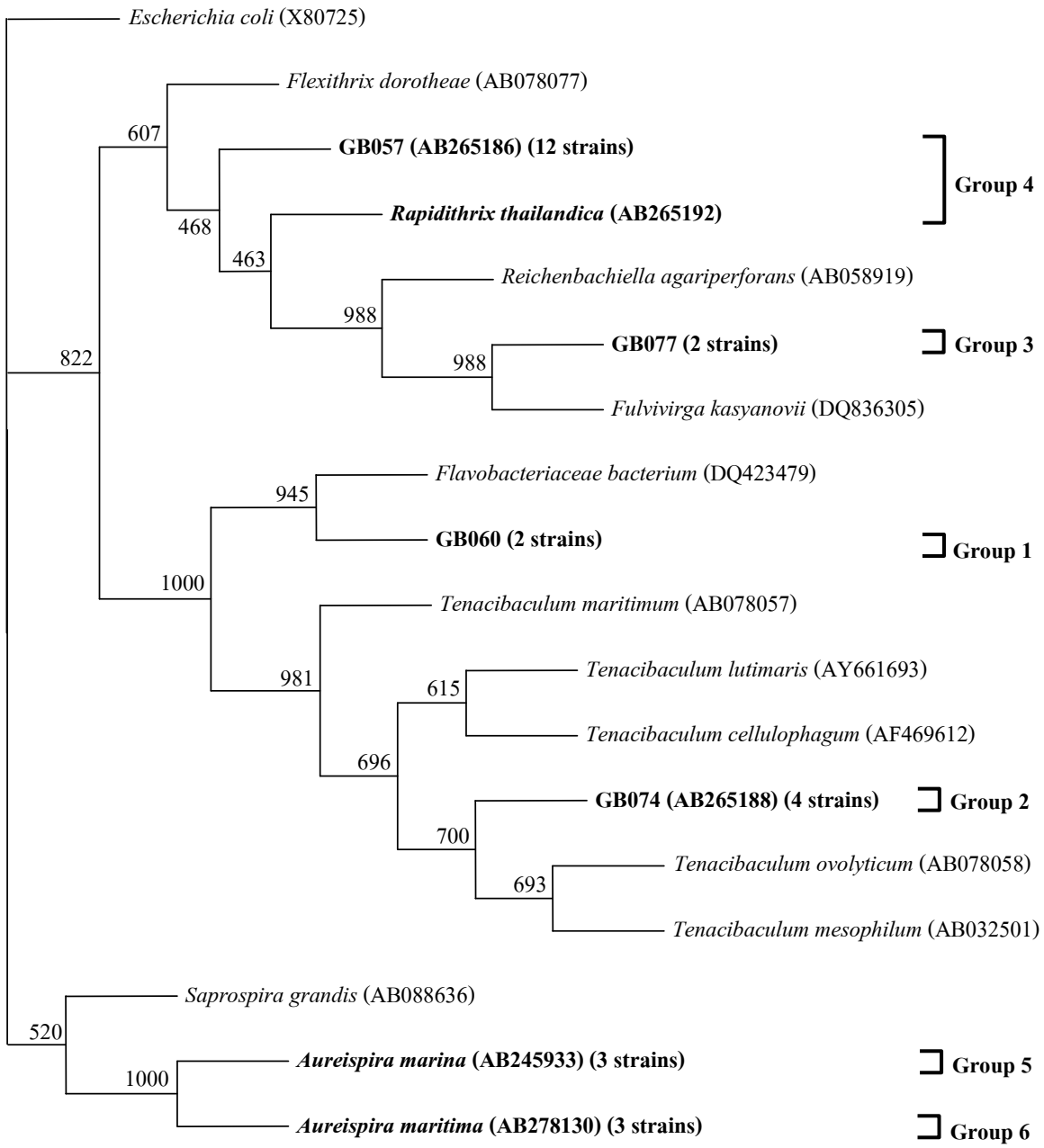


Figure 16. Maximum parsimony phylogenetic tree of marine gliding bacterial isolates and related members of the CFB group based on 16S rRNA gene sequence analysis using CLASTAL X version 1.83. Numbers within the phylogenetic tree indicate the percentages of occurrence of the branching order in 1000 bootstrapped trees

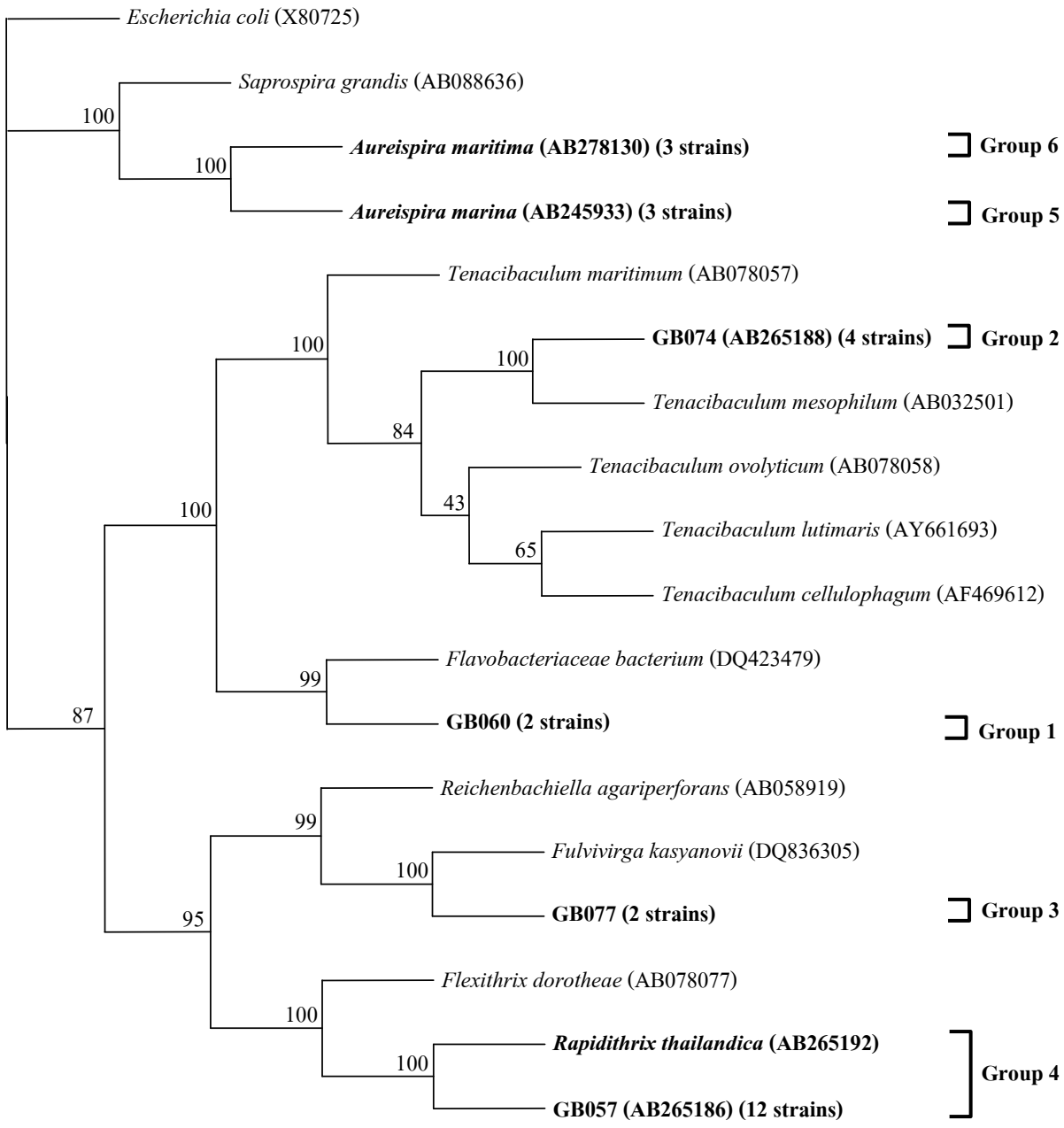


Figure 17. Maximum likelihood phylogenetic tree of marine gliding bacterial isolates and related members of the CFB group based on 16S rRNA gene sequence analysis using CLASTAL X version 1.83. Numbers within the phylogenetic tree indicate the percentages of occurrence of the branching order in 100 bootstrapped trees

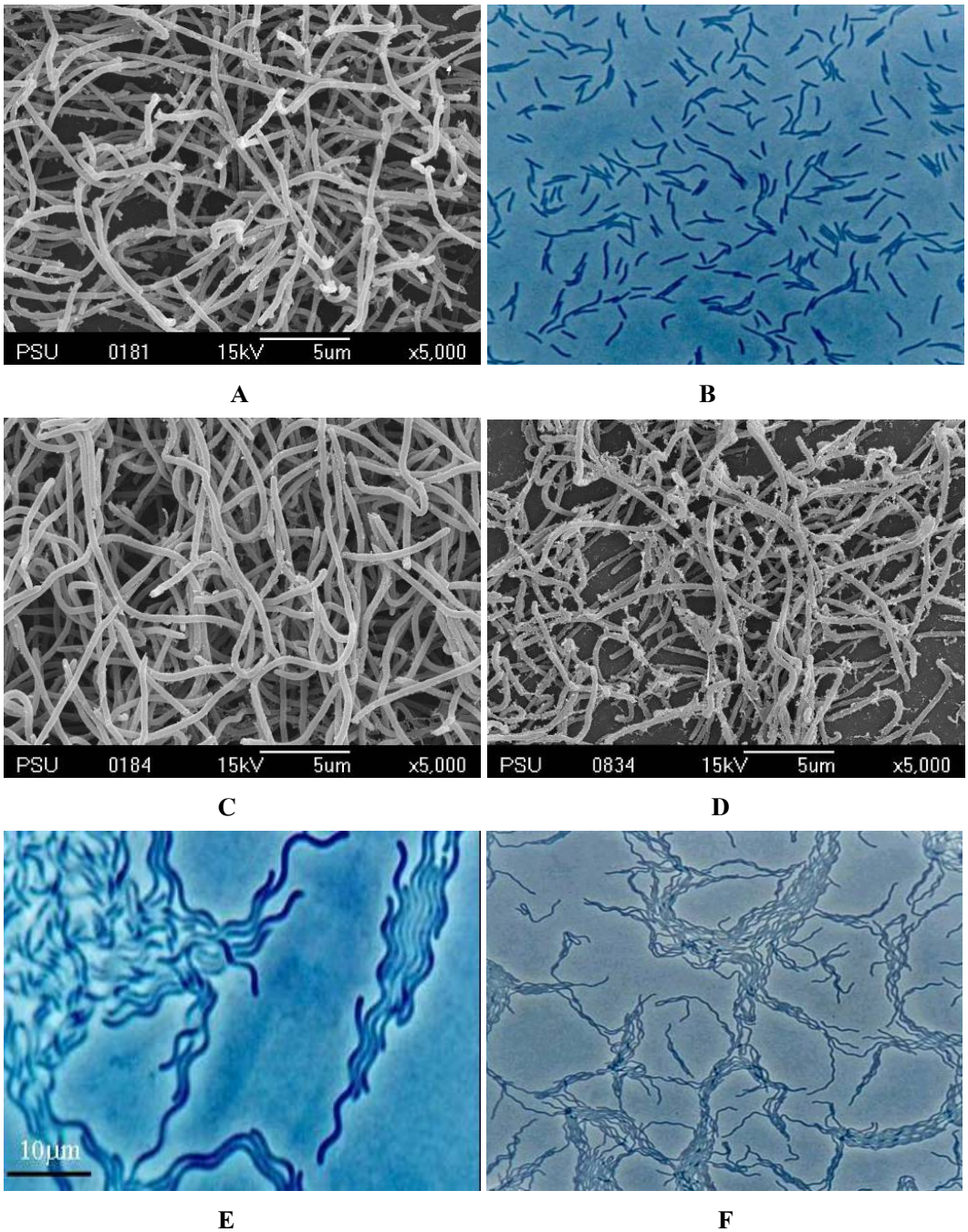


Figure 18. Cell morphology of isolates; (A) Scanning electron micrograph (×5,000) of candidate novel genus, GB060 or TISTR1763 (group 1). Bar = 5 µM; (B) Cell morphology of *Tenacibaculum mesophilum* (TISTR1755) under phase contrast microscope; (C) Scanning electron micrographs (×5,000) of TISTR1781 (group 3). Bar = 5 µM; (D) Scanning electron micrograph (×5,000) of *Rapidithrix thailandica* (TISTR1760). Bar = 5 µM; (E) Cell morphology of *Aureispira marina* (TISTR1719) under phase contrast microscope. Bar = 10 µM; (F) Cell morphology of *Aureispira maritima* (TISTR1726 = AB278130) under phase contrast microscope

Phylogenetic analysis of group 2 confirmed that all members of this group were *Tenacibaculum mesophilum*, a recently proposed marine gliding bacterium isolated from the surfaces of macroalgae by Suzuki *et al*., (2001). As the member of group 3, GB076 (TISTR1780) and GB077 (TISTR1781), showed 98% similarities with *Fulvivirga kasyanovii* (DQ836305) which was recently proposed by Nedashkovskaya *et al*. (2007). It might be possible that these could potentially be the novel species of the genus *Fulvivirga*. Normally, the percentage of similarity value between 98%-100% indicated the closest relative species. However, if the DNA-DNA relatedness between sample strain and type strains was below the currently accepted limit of DNA relatedness (70%), suggesting for the phylogenetic definition of a species (Stackebrandt and Goebel, 1994 referred by Choi *et al*., 2006). Several reports had previously proposed the novel species with 98% sequence similarities and DNA-DNA relatedness value below 70%. For instance, Frette and colleagues (2004) proposed a new species of *Tenacibaculum skagerrakense* which showed 98.1% and 97.7% sequence similarities with *T. mesophilum* and *T. amylolyticum*, respectively.

The 16S rRNA gene sequence similarity of group 4 and the closest species, *Flexithrix dorotheae* (AB078077) showed only 91%-92% similarity and the phylogenetic analysis of this group also formed a remote lineage from *Flexithrix dorotheae* (AB078077). These results indicated a novel species of a new genus, for which the name *Rapidithrix thailandica* gen. nov., sp. nov. was given (Srisukchayakul *et al*., 2007). It was described as unbranched and flexible filaments with the size of 0.7 × 20-100 μm. It was considered as chemo-organotrophs, strictly aerobes which require seawater for growth. The major repiratory quinone was MK-7 and the major cellular fatty acids 16:1 and 15:0. The G+C content was in the range 40-43 mol%. It also showed positive for oxidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, acid phosphatase, naphthol-AS-BI-phospho-hydrolase, *N*-acetyl-*β*-glucosaminidase and α-fucosidase. It, on the other hand, showed negative for catalase, lipase (C14), α-chymotrypsin, α-galactosidase, *β*-galactosidase, *β*-glucuronidase, α-glucosidase, *β*-glucosidase and α-mannosidase. It could also hydrolyze starch, gelatin, *L*-tyrosine, and tweens 20 and 80.

It is interesting to note that some of the members in this group i.e. GB057 (TISTR1760 = AB265186) and other 11 strains showed 97%-99% sequence similarity with the type strain *Rapidithrix thailandica* (AB265192) and the phylogenetic tree exhibited a separated lineage from the type strain (see Fig. 15, 16, 17). As previously stated, it might be possible that these twelve isolates belong to the separate species of the genus *Rapidithrix*. However, more descriptive details (i.e. DNA-DNA hybridization) are necessary to comfirm this assumption.

Phylogenetic analysis of group 5 formed a distinct lineage from the neighbor *Saprospira grandis* (AB088636) and their 16S rRNA gene sequences showed only 86% similarities with the closest neighbor. The members of this group were recently reported as a new genus for which the name *Aureispira marina* gen. nov., sp. nov. was proposed (Hosoya *et al*., 2006). The description of this novel genus and species included an unbranched cell (0.8-1.2 × 1.5-2.5 μm) with spiral shape and gold color colony, formed flexible helical filaments up to 100 μm (helix width 1.5-2.0 μm, helix pitch 4-9 μm), strictly aerobic, required seawater for growth. The optimal temperature was 25-30 °C and the optimal pH was 6.0-8.0. The G+C content was in the range 38-39 mol%. The major respiratory quinone was MK-7. It was important to note that the predominant cellular fatty acids were 20:4ω6*c* (arachidonic acid), 16:0 and iso-17:0 which was unusual for prokaryote.

Lastly, the 16S rRNA genes of group 6 showed 94% similarities with *Aureispira marina* and its phylogenic analysis also formed a separating lineage from this strain. Based on the genetic relatedness analysis which was done by our research group, the DNA-DNA relatedness between group 6 and *A. marina* was less than 5% suggested that this group should be classified to new species as *Aureispira maritima* sp. nov. (Hosoya *et al*., 2007). The description of this new species includes spiral cell shape with gold color colony. The G+C content was 38.7 mol% and the major respiratory quinone was MK-7. The predominant cellular fatty acids were 20:4ω6*c* and 16:0.

Cell morphology of each marine gliding bacterial group is shown in Fig. 18. Based on these results, it cleary indicated that the biodiversity of marine gliding bacteria in Thailand is different from what has been reported recently in the other geographical locations. For instances, *Cellulophaga baltica* and *C. fucicola* (Johansen *et al*., 2001), *Zobellia galactanovorans* (Barbeyron *et al*., 2001), *Reichenbachia agariperforans* (Nedashkovskaya *et al*., 2003),

Psychroflexus tropicus (Donachie *et al*., 2004), *Tenacibaculum aestuarii* (Jung *et al*., 2006), *T. litopenaei* (Sheu *et al*., 2007) which were isolated mostly in the temperate zones.

It is important to state that no myxobacteria (non-fruiting forming gliding bacteria) was isolated in this study eventhough several investigators had reported the isolation of marine myxobacteria from the South China Sea and Pacific Ocean (Iizuka *et al*., 2003a; Iizuka *et al*., 2003b; Li *et al*., 2002; Iizuka *et al*., 1998). This might be the result of the differences in isolation media, conditions and specimens used for the isolation. It would be interesting, however, to expand the isolation schemes i.e. isolation media components, salinity as well as the use of baiting technique in order to increase the possibility of obtaining different groups of marine gliding bacteria.

3.3 Cytotoxicity screening

In this study, forty-two isolates out of total eighty-four isolates of marine gliding bacterial cultures were used to screen for cytotoxicity. The total of 168 crude extracts, obtained from the cultivation of 42 selected strains in four different cultivation media, were submitted for cytotoxicity assay against four cancer cells lines including HeLa, HT-29, KB and MCF-7 using SRB method as described earlier. All of the crude extracts were diluted to the final concentration of 25 μg/ml with the DMSO. The extracts showed the growth inhibition of one or more cancer cell lines greater than 80 percents was considered cytotoxicity (Table 5). These results showed that 28 out of 168 crude extracts, obtained from different media, have cytotoxicities. The result (Table 6) showed that 14 out of 42 tested isolates (33.3%) could produce the crude extract which exhibited cytotoxicity. It is interesting to note that the members of group 2 (*Tenacibaculum mesophilum*) and group 4 (*Rapidithrix thailandica*) were capable of producing metabolites with cytotoxicities against all four cell lines, whereas only one member of group 6 (*Aureispira maritima*) and unidentified group produced metabolites against only HeLa and HT-29. In contrast, group 1 (candidate novel genus), group 3 (*Fulvivirga kasyanovii*) and group 5 (*Aureispira marina*) exhibited no cytotoxicity. Among these, all the members of *Tenacibaculum mesophilum* (gr. 2) were capable of producing the crude extracts with cytotoxicity against all cell lines. However,

there has been no report or evidence on secondary metabolites from *T. mesophilum* and it would be worth investigating their metabolites.

Table 5. Cytotoxicity of crude extracts (conc. 25 µg/ml) against HeLa, HT-29, KB and MCF-7 cell lines

Tested strain	**Cultivation media**	**% Inhibition**			
		HeLa	**HT-29**	**KB**	**MCF-7**
Tenacibaculum mesophilum (TISTR1755)	CY	**89.7**	**89.8**	**80.6**	**85.0**
Tenacibaculum mesophilum (TISTR1778)	CY	**81.2**	73.3	52.4	2.6
	SK	31.5	**85.9**	44.2	15.1
Tenacibaculum mesophilum (TISTR1782)	RL 1	74.7	**91.0**	**90.7**	52.6
	CY	60.6	**92.2**	**89.3**	33.1
	SK	65.0	**93.3**	**92.1**	40.2
Tenacibaculum mesophilum (TISTR1784)	CY	**86.8**	**91.7**	**89.5**	**94.1**
	SK	**81.1**	**94.2**	**93.3**	69.4
Rapidithrix thailandica (TISTR1741)	Vy/2	**87.4**	**88.6**	75.8	55.5
	RL 1	76.3	**84.3**	75.3	50.4
	CY	41.0	**86.1**	57	29.5
Rapidithrix thailandica (TISTR1747)	CY	17.1	**87.3**	31.1	17.8
Rapidithrix thailandica (TISTR1749)	RL 1	18.5	**88.9**	34.5	23.4
	CY	33.3	**84.3**	53.7	33.6
Rapidithrix thailandica (TISTR1750)	Vy/2	40.5	**85.4**	36.9	20.8
	CY	**88.4**	**97.4**	**99.8**	66.1
Rapidithrix thailandica (TISTR1760)	SK	21.0	**81.1**	46.0	-54.5
Rapidithrix thailandica (TISTR1772)	RL 1	40.9	**85.5**	62.2	17.4
	SK	23.8	**80.2**	45.7	-7.7
Rapidithrix thailandica (TISTR1768)	CY	46.3	**81.6**	51.2	61.3
Rapidithrix thailandica (TISTR1785)	Vy/2	**91.4**	**91.4**	**99.4**	69.7
	RL 1	**91.4**	79.2	**87.6**	60.5
	CY	**99.5**	**98.2**	**99.9**	**82.7**
	SK	**96.9**	**96.0**	**99.8**	78.2
Aureispira maritima (TISTR1726)	Vy/2	21.2	**85.6**	41.7	23.1
	CY	**84.2**	-12.8	18.6	-15.4
Unidentified strain (TISTR1746)	RL 1	69.7	**83.7**	71.8	43.1

Eight strains of group 4 (*Rapidithrix thailandica*) could produce metabolites which inhibited HT-29. Whereas only one could produced compound with cytotoxicity against MCF-7. This indicated that marine gliding bacteria could be as good source of cytotoxic compounds especially the genera *Rapidithrix* and *Tenacibaculum.*

Table 6. Cytotoxicity of crude extracts obtained from each taxonomic group

Group of isolates	**Number of tested isolates (*)**	**Number of isolates producing cytotoxic activities on**			
		HeLa	**HT-29**	**KB**	**MCF-7**
Group 1 (Candidate for novel genus)	2 (0)	0	0	0	0
Group 2 (*Tenacibaculum mesophilum*)	4 (4)	3	4	3	2
Group 3 (*Fulvivirga kasyanovii*)	2 (0)	0	0	0	0
Group 4 (*Rapidithrix thailandica*)	13 (8)	3	8	2	1
Group 5 (*Aureispira marina*)	3 (0)	0	0	0	0
Group 6 (*Aureispira maritima*)	3 (1)	1	1	0	0
Unidentified	15 (1)	0	1	0	0
Total	42 (14)	7	14	5	3

Note: (*) number of active isolates

It was also important to note that compositions of cultivation media played an important role on the production of the cytotoxic metabolites. In this study CY medium was the most productive medium for cytotoxic metabolites production, yielding 23 cytotoxic extracts, followed by SK (11 extracts), RL1 (8 extracts) and Vy/2 (7 extracts) as shown in Figure 19. Moreover, only CY medium could yield the crude extracts which inhibited all four cell lines. Whereas other media could not produce crude extract which inhibited MCF-7. Casitone (pancreatic digest of casein), which was present only in CY medium, was described as an important promotor to produce protease enzyme in microorganisms such as *Aspergillus oryzae*

U1521 (Samarntarn *et al*., 1999) and *Lactococcus lactis* BGIS29 (Miladinov *et al*., 2001). Samarntarn and co-workers (1999) described that amino acids and/or peptides in casitone may be served as the specific inducers for increasing the total alkaline protease production. The rich of amino acids and/or peptides in CY medium which could derive from casitone might then cause the increasing of cytotoxic compound production. This is the first report on the screening for cytotoxicity of marine gliding bacteria.

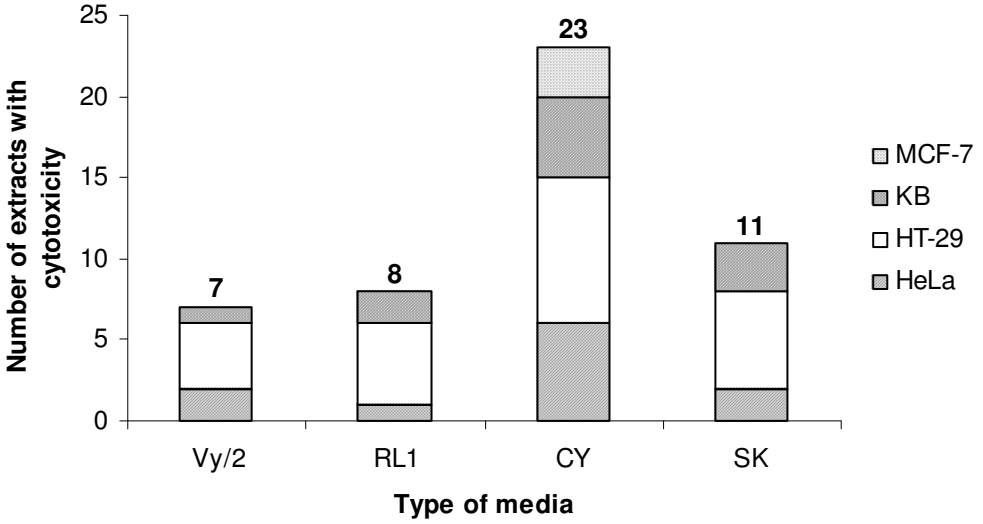


Figure 19. Effect of media types on cytotoxic activity

3.4 Isolation and structure elucidation of cytotoxic compounds

The bioassay guided fractionation of the crude extracts led to the discovery of several metabolites including both previously reported and novel compounds. Base on the cytotoxicity of the crude extracts, gliding bacterial strains including *Rapidithrix thailandica* (GB064 = TISTR1768), *Rapidithrix thailandica* (TISTR1741 = AB265183) and *Tenacibaculum mesophilum* (GB078 = TISTR1782 = AB265189) were chosen for further investigation with the procedures described previously in Chapter 2.

3.4.1 Compounds G1-G4

An aliquot of crude extract from *Rapidithrix thailandica* strain GB064 (3.6 g) was subjected to a size exclusion chromatography on a Sephadex LH-20 column using methanol as an eluent to yield five fractions (CY001-CY005). Fraction CY004 (541.1 mg, 83.3 % inhibition to HT-29 at concentration of 25 ug/ml) was further separated using a semi-preparative RP C-18 HPLC (Phenomenex®; 250×10 mm, 10 µm) with 30:70 methanol in water as a mobile phase (4.5 ml/min; 210 nm), from which compounds **G1-G4** were obtained (t_R = 20, 23, 28 and 31 min, respectively). The isolation scheme of compounds **G1-G4** is shown in Scheme 1.

The molecular formula of compound **G1** was deduced as $C_{14}H_{16}N_2O_2$ from the mass spectrum (*m/z* 243.8). The ^{1}H NMR data (500 MHz, DMSO-d_6) exhibited five proton resonances at δ 7.13 (dd; *J* = 1.8, 8.2 Hz; H-12/16), 7.27 (dd; *J* = 1.8, 4.0 Hz; H-14) and 7.28 (dd; *J* = 1.4, 5.6 Hz; H-13/15) indicated the presence of one aromatic ring (Table 7). This corresponded well with the ^{13}C NMR spectrum which showed four signals at δ 127.1 (C-14), 128.5 (C-13/15), 130.0 (C-12/16) and 136.3 (C-11). In addition, two carbonyls at δ 165.0 (C-2) and 168.6 (C-5) were observed. The presence of a secondary amine was deduced from the characteristic IR absorption band at ν_{max} 3250 cm^{-1}, and from the exchangeable proton resonance at δ 8.19 (br d; *J* = 3.5 Hz). The remaining carbons including four methylenes (δ 21.6, 28.7, 39.4 and 44.9) and two methines (δ 57.4, 58.2) were deduced from DEPT spectra. The COSY spectrum delineated the partial structures of H-10a (δ 2.89, dd; *J* = 5.2, 13.4 Hz), H-10b (δ 3.02, dd; *J* = 5.8, 13.4 Hz), and H-3 (δ 3.98, dd; *J* = 5.2, 5.8), and of H-6 (δ 2.92, dd; *J* = 6.4, 6.6), H-7 (δ 1.60 m, 1.92 m), H-8

(δ 1.58 m, 1.78 m), and H-9a (δ3.20 m), H-9b (δ3.41 m). Linking the two partial structures and the phenyl moiety was carried out by means of HMBC spectral analysis. Correlations from C-11 to H-3 and H-10 helped connecting the phenyl ring onto C-10, whereas those from C-2 to H-3, and from C-5 to H-6 suggested the 2,5-diketopiperazine moiety. The structure of **G1** was therefore proposed to be cyclo (L-Pro-D-Phe) as shown in Figure 20 (Stierle *et al*., 1988).

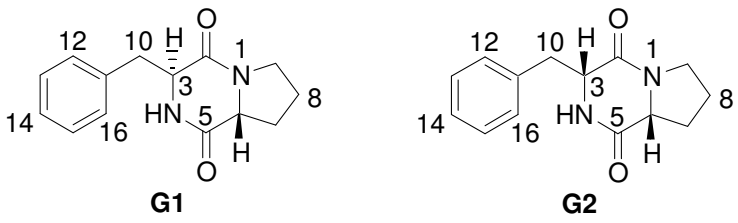


Figure 20. Chemical structures of **G1** and **G2**

Table 7. ^{1}H and ^{13}C NMR spectral data of compounds **G1** amd **G2** (500 MHz for ^{1}H; DMSO-d_6)

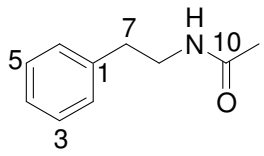
Position	δ_H (mult.; J in Hz)[a]		δ_C (mult.)		
	G1	**G2**	**G1**	**G2**	
2	-	-	165.0	165.3	(C)
3	3.98 (dd; 5.2, 5.8)	4.34 (dd; 4.9, 5.0)	58.2	55.9	(CH)
4-NH	8.19 (br d; 3.5)	8.02 (br s)	-	-	
5	-	-	168.6	169.3	(C)
6	2.92 (dd; 6.4, 6.6)	4.06 (dd; 7.2, 8.8)	57.4	58.6	(CH)
7a	1.60 (m)	1.72 (m)	28.7	28.0	(CH_2)
b	1.92 (m)	2.00 (m)			
8a	1.58 (m)	1.39 (m)	21.6	22.1	(CH_2)
b	1.78 (m)	1.68 (m)			
9a	3.20 (m)	3.25 (m)	44.9	44.8	(CH_2)
b	3.41 (m)	3.40 (m)			
10a	2.89 (dd; 5.2, 13.4)	2.98 (dd; 4.9, 14.2)	39.4	35.5	(CH_2)
b	3.02 (dd; 5.8, 13.4)	3.04 (dd; 5.0, 14.2)			
11	-	-	136.3	137.5	(C)
12/16	7.13 (dd; 1.9, 8.4; 2H)	7.25 (m; 2H)	130.0	130.0	(CH; 2C)
13/15	7.28 (dd; 2.5, 8.4; 2H)	7.25 (m; 2H)	128.5	128.2	(CH; 2C)
14	7.27 (dd; 1.9, 8.6)	7.18 (m)	127.1	126.6	(CH)

Note: a) unless stated otherwise, each proton signal was integrated as 1 H

The identical molecular mass observed at *m/z* 243.8 in the EI mass spectrum and the close similarity between the NMR spectra between these of compounds **G1** and **G2** suggested that the two compounds are isomer. The major differences between both compounds were observed at the resonances of H-3 and H-10. The up-fielded shift of the resonance for H-3 (δ 4.34, dd; J = 4.9, 5.0 Hz) and the less resolved resonances for H-10a (δ 2.98, dd; J = 4.9, 14.2

Hz) and H-10b (δ 3.04, dd; J = 5.0, 14.2 Hz) assigned for **G2** indicated that both compounds differed primarily within regards of C-3 configuration; therefore **G1** and **G2** are epimers. As the nOe enhancement observed between H-3 and H-6 indicating that the two protons adopt the same plane, compound **G2** therefore was proposed as syn epimer of cyclo(L-Pro-L-Phe) (Furtado *et al*., 2005); hence, compound **G1** was its anti analog. The specific rotations of compounds **G1** and **G2** are +95.7 (*c* 0.06; MeOH) and –93.9 (*c* 0.1; EtOH) (Seto *et al*., 2005), respectively, also confirmed the proposed structure (Fig. 20).

The EI mass spectrum (*m/z* 163.0) of compound **G3** was deduced to give the molecular formula as $C_{10}H_{13}NO$. The ^{1}H NMR data (500 MHz, $CDCl_3$+CD_3OD) (Table 8) exhibited the characteristic resonances of a monosubstituted phenyl moiety at δ7.21 (dd; J = 1.3, 8.4 Hz; H-2/6), 7.25 (tt; J = 1.3, 7.4 Hz; H-4) and 7.32 (dd; J = 1.7, 7.9 Hz; H-3/5). The presence of a secondary amide moiety was deduced from the IR absorption band at ν_{max} 3280 cm^{-1} and 1650 cm^{-1}, and from the NMR resonances at δ_C 170.9 (C-10) and δ_H 6.24 (br s; H-9). On one hand, a methyl group (δ1.94 s; H-11) was attached to the carbonyl C-10. On the other hand, an ethylene bridge of H-7 (δ2.82, t; J = 7.0 Hz) and H-8 (δ3.49, dt; J = 5.8, 7.0 Hz) was used to connect the phenyl and amide moiety. The structure of compound **G3** (Shaaban *et al*., 2002) was therefore proposed as *N*-(2-phenylethyl) acetamide (Fig. 21).



G3

Figure 21. Chemical structure of compound **G3**

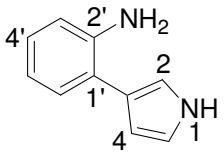
Table 8. ^{1}H and ^{13}C NMR spectral data of compound **G3** (500 MHz for ^{1}H; $CDCl_3$+CD_3OD)

Position	δ_H (mult.; *J* in Hz)	δ_C (mult.)
1	-	138.8 (C)
2/6	7.21 (dd; 1.3, 7.8; 2H)	128.6 (CH; 2C)
3/5	7.32 (dd; 7.4, 7.8; 2H)	128.5 (CH; 2C)
4	7.25 (tt; 1.3, 7.4)	126.4 (CH)
7	2.82 (t; 7.0)	35.3 (CH_2)
8	3.49 (dt; 5.8, 7.0)	40.5 (CH_2)
9	-	-
10	-	170.9 (C)
11	1.94 (s)	22.8 (CH_3)

The low resolution EI mass spectrum of compound **G4** gave the molecular signal at *m/z* 157.9, corresponding to the molecular formula $C_{10}H_{10}N_2$. The ^{1}H NMR spectrum (500 MHz, $CDCl_3$) (Table 9) showed seven resonance signals, assigned to two aromatic spin systems. The signals at δ6.76 (dd; *J* = 1.2, 7.7 Hz; H-3′), 6.79 (ddd; *J* = 1.2, 7.5, 7.7 Hz; H-5′), 7.08 (ddd; *J* = 1.2, 7.5, 7.7 Hz; H-4′) and 7.25 (dd; *J* = 1.2, 7.7 Hz; H-6′) were characterized as a 1,2-disubstituted benzene. On the other hand, the resonance signals at δ6.44 (ddd; *J* = <1, 2.3, 2.3 Hz; H-4), 6.85 (ddd; *J* = 2.3, 2.3, 2.3 Hz; H-5) and 6.96 (ddd; *J* = <1, 2.3, 2.3 Hz; H-2) were constituted to a 3-substituted pyrrole moiety. HMBC correlations from C-3 (δ 121.8) to H-6′ suggested the benzene-pyrrole connection. The ^{1}H NMR spectrum exhibited two broad exchangeable protons at δ 3.97 and 8.39 which were assigned to the secondary amino (2′-NH_2) and the pyrrole proton (H-1), respectively.

The structure of compound **G4** was therefore proposed as 3-(2'-aminophenyl)-pyrrole. The nOe enhancements between each pair of protons along the circumference of the structure, i.e., H-1 ↔ H-2 ↔ NH_2-2' ↔ H-3' ↔ H-4' ↔ H-5' ↔ H-6' ↔ H-4 ↔ H-5 ↔ H-1, supported the proposed structure (Fig. 22). Compound **G4** was reported here as a natural

product; however, its structure had been theoretically proposed as one of biosynthetic intermediates of the antibiotic pyrrolnitrin previously reported in many Pseudomonads such as *Pseudomonas cepacia* B37w (Burkhead *et al*., 1994), *Pseudomonas fluorescens* (Kirner *et al*., 1998), *Pseudomonas pyrrocinia*, *Pseudomonas aureofaciens* (Lee *et al*., 2005) and *Pseudomonas* sp. MF381-IODS (Pohanka *et al*., 2005).



G4

Figure 22. Chemical structure of compound **G4**

Table 9. ^{1}H and ^{13}C NMR spectral data of compound **G4** (500 MHz for ^{1}H; $CDCl_3$)

Position	δ_H (mult.; *J* in Hz)	δ_C (mult.)		HMBC correlation (C ⟶ H)
1-NH	8.39 (br s)		-	-
2	6.96 (ddd; <1,2.3,2.3)	116.1	(CH)	H-4, H-5
3	-	121.8	(C)	H-2, H-4, H-5, H-6′
4	6.44 (ddd; <1,2.3,2.3)	108.6	(CH)	H-2, H-5
5	6.85 (ddd; 2.3,2.3,2.3)	118.4	(CH)	H-2, H-4
1′	-	122.2	(C)	H-3′, H-4′, H-5′, H-6′
2′	-	143.7	(C)	H-4′, H-5′, H-6′
3′	6.76 (dd; 1.2,7.7)	115.5	(CH)	H-5′
4′	7.08 (ddd; 1.2,7.5,7.7)	127.1	(CH)	H-3′, H-5′
5′	6.79 (ddd; 1.2,7.5,7.7)	118.6	(CH)	H-3′, H-4′, H-6′
6′	7.25 (dd; 1.2,7.7)	129.8	(CH)	H-4′, H-5′
2′-NH_2	3.97 (br s; 2H)		-	-

Note: Broad singlest signal means a 1 proton

Pyrrolnitrin is a tryptophan-derived secondary metabolite with broad-spectrum antifungal activity. Pyrrolnitrin production in *Enterobacter agglomerans*, were also reported *Myxococcus fulvus*, *Corallococcus exiguous*, *Cystobacter ferrugineus* (Gerth *et al*., 1982 referred by Laurentis *et al*., 2006). The pyrrolnitrin biosynthetic gene cluster from *Pseudomonas fluorescens* (BL915) was finally identified by van Pee and co-workers in 1997 (referred by Lee *et al*., 2005; Laurentis *et al*., 2006). This led to a proposal of the pathway for pyrrolnitrin biosynthesis (Fig. 23). The first and third steps are catalyzed by two related enzymes, PrnA and PrnC. Both PrnA and PrnC belong to the flavin-dependent halogenase superfamily (Laurentis *et al*., 2006). In this pathway, the first step is the chlorination of tryptophan by PrnA at the 7 position to form 7-chlorotryptophan, followed by rearrangement of the indole ring to a phenylpyrrole ring and decarboxylation by PrnB to form monodechloroaminopyrrolnitrin. This intermediate is chlorinated a second time by PrnC to form aminopyrrolnitrin, which, in the last step of the pathway, undergoes oxidation of the amino group in aminopyrrolnitrin to a nitro group by PrnD to form pyrrolnitrin (Lee *et al*., 2005).

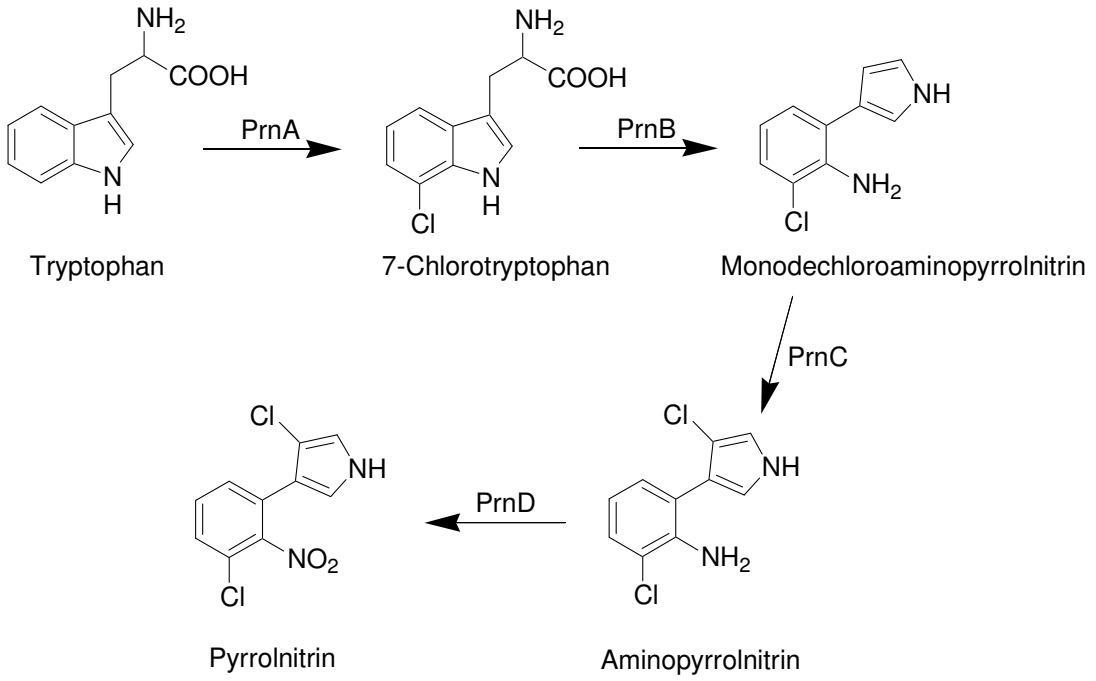


Figure 23. The biosynthetic pathway of pyrrolnitrin

Source: Laurentis *et al*. (2006)

3.4.2 Compounds G5 and G6

The remaining of fraction CY015 (225.2 mg) was pooled and further separated using an RP C-18 HPLC (Phenomenex; 250×10 mm, 10 μm; 15:85 methanol in water, 4.5 ml/min; 210 nm). Further isolation on a SiO_2 column using a mixture of methanol and dichoromethane (5:95) as mobile phase yielded compounds **G5** (7.7 mg) and **G6** (5.8 mg) (Scheme 1).

The molecular formula of compound **G5** was deduced as $C_{14}H_{16}N_2O_3$ from the EI mass spectrum (*m/z* 260.0). The additional oxygen atom and the close similarity among the spectral data of **G1**, **G2** and **G5** indicated that all were analog. With the characteristic resonances of *p*-substituted phenyl moiety as seen in the ^{1}H and ^{13}C NMR spectra (δ_H 7.07, d; J = 8.2 Hz; H-12/16; 6.79, d; J = 8.3 Hz; H-13/15 and δ_C 127.2, C-11; 130.3, C-12/16; 116.1, C-13/15; 155.4, C-14) (Table10), compound **G5** was therefore proposed as a tyrosinyl analog of **G1** and **G2** (Fig. 24).

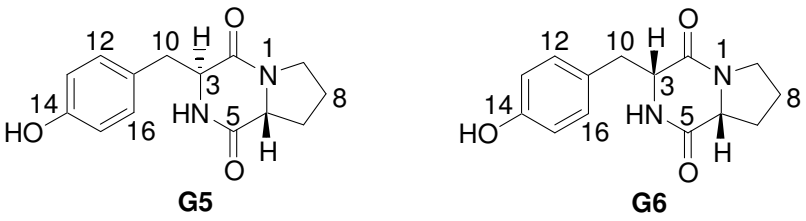


Figure 24. Chemical structures of compounds **G5** and **G6**

Table 10. ^{1}H and ^{13}C NMR spectral data of compounds **G5** and **G6** (500 MHz for ^{1}H; $CDCl_3$ for **G5** and DMSO-d_6 for **G6**)

Position	δ_H (mult.; J in Hz)[a]		δ_C (mult.)		
	G5	**G6**	**G5**	**G6**	
2	-	-	165.1	165.0	(C)
3	4.22 (dd; 3.3, 10.4)	3.91 (dd; 3.8, 7.8)	56.2	57.3	(CH)
4-NH	5.78 (br s)	8.12 (br d; 3.7)	-	-	
5	-	-	169.6	168.5	(C)
6	4.09 (ddd; <1, 7.5, 7.7)	3.31 (m)	59.1	58.3	(CH)
7a	2.34 (m)	1.54 (m)	28.8	28.7	(CH_2)
b	2.01 (m)	1.59 (m)			
8a	1.90 (m)	1.93 (dd; 5.1, 12.1)	22.5	21.4	(CH_2)
b	2.04 (m)	2.74 (dd; 5.1, 13.7)			
9a	3.56 (m)	2.92 (dd; 5.0, 5.5)	45.4	44.7	(CH_2)
b	3.62 (m)	3.20 (m)			
10a	2.75 (dd; 10.4, 14.6)	2.83 (dd; 7.8, 10.6)	35.9	38.8	(CH_2)
b	3.50 (dd; 3.3, 14.6)	3.18 (dd; 3.8, 10.6)			
11	-	-	127.2	126.0	(C)
12/16	7.07 (d; 8.3; 2H)	6.88 (d; 8.2; 2H)	130.3	130.9	(CH; 2C)
13/15	6.79 (d; 8.3; 2H)	6.65 (d; 8.2; 2H)	116.1	115.2	(CH; 2C)
14	-	-	155.4	156.5	(C)
17-OH	-	9.34 (br s)	-	-	

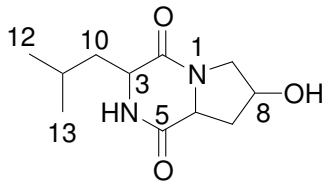
Note: a) unless stated otherwise, each proton signal was integrated as 1 H

Similar to **G1** and **G2**, **G6** was found to possess similar spectral data to that of **G5**. The major differences between **G5** and **G6** were observed around the resonances of H-3 (δ 3.91, dd; J = 3.8, 7.8 Hz), H-10a (δ 2.83, dd; J = 7.8, 10.6 Hz), and H-10b (δ 3.18, dd; J = 3.8, 10.6 Hz) (Table 10). The chemical structure of **G6** (Fig. 24) was therefore proposed to an epimer of **G5**. The nOe enhancement between H-3 and H-6 indicated that **G6** was a *cis*-epimer of **G5**. It is worth mentioning here that, whereas all the nOe experiments, either with **G5** or **G6**, strongly confirmed the proposed relative configuration, the ^{1}H NMR spectra between the **G1** and **G2** and the **G5** and **G6** were opposite.

3.4.3 Compounds G7 and G8

An aliquot (2.8 g) from the crude extract of *Tenacibaculum mesophilum* (strain GB078 = TISTR1782 = AB265189) was subjected to a Sephadex LH-20 column using methanol as an eluent. The fraction RL002 (106.9 mg) was separated over an RP C-18 HPLC column (Phenomenex, 250×10 mm, 10 µm) 30:70 methanol in water as a mobile phase (3.5 ml/min, 210 nm) and compounds **G7** (3.3 mg) and **G8** (5.1 mg) was obtained (t_R = 10 and 13 min, respectively). The isolation scheme of compounds **G7** and **G8** is shown in Scheme 2.

The ESI mass spectrum of compound **G7** showed the pseudomolecular signal of [M+Na^{+}] at *m/z* 249.6, indicating to the molecular formula $C_{11}H_{18}N_2O_3$. Based on the ^{13}C NMR data, the presence of two methyls, three methylenes, four methines, and two quaternary carbons were observed (Table 11). The presence of a secondary amine was observed at the IR absorption band (ν_{max} 3250 cm^{-1}). The ^{13}C and ^{1}H NMR spectra showed similar pattern of a diketopiperazine moiety similar to those found with compounds **G1**, **G2**, **G5** and **G6**. The proline residue as formerly seen in compounds **G1**, **G2**, **G5** and **G6**, however, is replaced here with a γ-hydroxy proline as the resonances for a carbonol moiety resonating at δ_C 67.8 (C-8) and δ_H 4.45 (s; H-8) were observed. On the other hand, the residues of phenylalanine as seen in **G1** and **G2** and of tyrosine as seen in **G5** and **G6** are also replaced with a leusine. The characteristic *iso*-butyl side chain was observed at δ_H 1.63 (dd; J = 3.9, 3.9; H-10), 1.78 (m; H-11), 0.95 (d; J = 3.9; H-12), 0.99 (d; J = 3.9; H-13). Therefore, **G7** was proposed as cyclo(8-hydroxy-L-Pro-L-Leu) (Fig. 25) (Rosa *et al*., 2003).



G7

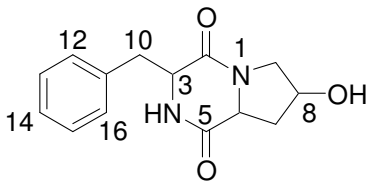
Figure 25. Chemical structure of compound **G7**

Table 11. ^{1}H and ^{13}C NMR spectral data of compound **G7** (300 MHz for ^{1}H; $CDCl_3$+CD_3OD)

Position	δ_H (mult.; *J* in Hz)	δ_C (mult.)
2	-	167.5 (C)
3	3.93 (m)	56.1 (CH)
4-NH	-	-
5	-	169.4 (C)
6	4.18 (s)	56.4 (CH)
7a	2.41 (m)	36.7 (CH_2)
b	2.46 (m)	
8	4.45 (s)	67.8 (CH)
9a	3.40 (d; 5.7)	53.6 (CH_2)
b	3.82 (d; 7.2)	
10	1.63 (dd; 3.9, 3.9)	42.2 (CH_2)
11	1.78 (m)	24.5 (CH)
12	0.95 (d; 3.9)	21.4 (CH_3)
13	0.99 (d; 3.9)	22.9 (CH_3)

The low resolution ESI mass spectrum of compound **G8** showed the signal of $[M+Na]^+$ at *m/z* 283, corresponding to the molecular formula $C_{14}H_{16}N_2O_3$. The similar molecular formula and NMR spectral data among **G5**, **G6** and **G8** (Table 12) are again pronounced. Here, the major differences among all three diketopiperazine analogs were at the hydroxyl substitution sites. The characteristic resonances of monosubstituted phenyl moiety were observed at δ_H 7.20

(dd; J = 1.2, 8.1 Hz; H-12/16), 7.31 (m; H-13/15) and 7.33 (m; H-14), and a carbonol signal at δ_H 4.31 (m; H-8) indicated that **C-8** contains phenyl alanine and hydroxyl proline residues instead of tyrosine and proline ones as in **G5** and **G6**. Here, **G8** is proposed as cyclo(8-hydroxy-L-Pro-D-Phe) (Fig. 26) (Rosa *et al*., 2003).



G8

Figure 26. Chemical structure of compound **G8**

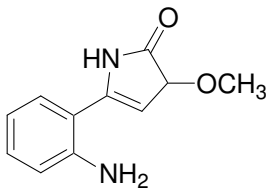
Table 12. ^{1}H and ^{13}C NMR spectral data of compound **G8** (300 MHz for ^{1}H; $CDCl_3$+CD_3OD)

Position	δ_H (mult.; J in Hz)	δ_C (mult.)
2	-	165.8 (C)
3	4.24 (m)	58.8 (CH)
4-NH	6.83 (br s)	-
5	-	169.1 (C)
6	2.96 (dd; 4.8, 4.8)	55.9 (CH)
7a	2.24 (m)	37.0 (CH_2)
b	2.35 (m)	
8	4.31 (m)	67.6 (CH)
9a	3.29 (dd; 3.3, 7.5)	53.3 (CH_2)
b	3.76 (dd; 1.7, 7.5)	
10a	3.05 (dd; 2.4, 8.1)	40.1 (CH_2)
b	3.15 (dd; 3.6, 8.1)	
11	-	135.2 (C)
12/16	7.20 (dd; 1.2, 8.1; 2H)	129.9 (CH; 2C)
13/15	7.31 (m; 2H)	128.8 (CH; 2C)
14	7.33 (m)	127.5 (CH)

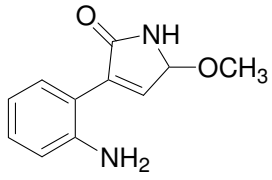
3.4.4 Compound G9

Crude extract (0.9 g) from the fermentation broth of marine gliding bacterium *R. thailandica* (TISTR1741 = AB265183) in modified Vy/2 medium was subjected to a size exclusion chromatography on a Sephadex LH-20 column (methanol), then to an RP C-18 HPLC (Phenomenex, 250×10 mm, 10 µm, 30:70 MeOH in H_2O, 4.5 ml/min, 210 nm) to yield compounds **G9** (1.2 mg); t_R = 15 min (Scheme 3).

The molecular formula of compound **G9** was proposed as $C_{11}H_{12}O_2N_2$, according to the pseudomolecular signal at *m/z* 205.1 $[M+H]^+$. This was confirmed by the pseudomolecular signal at *m/z* 205.0970 $[M+H]^+$ (calc for $C_{11}H_{12}O_2N_2$ 205.0974) in ESIMS spectrum. The ^{13}C NMR spectrum exhibited signal of four sp^2 methines (δ 117.1, C-3′; 130.5, C-4′; 118.7, C-5′ and 130.6, C-6′) and two quarternary carbons (δ 116.9, C-1′; and 145.4, C-2′), among these six belonged to a phenyl moiety. From the ^1H NMR spectrum (500 MHz, $CDCl_3$) (Table 13), a series of resonances assigned to an *o*-disubstituted phenyl moiety were observed at δ 6.73 (d; *J* = 8.0 Hz; H-3′), 7.18 (dd; *J* = 7.0, 8.0 Hz; H-4′), 6.78 (dd; *J* = 7.0, 7.5 Hz; H-5′) and 7.28 (m; H-6′). A primary amino group was proposed to attach to C-2′ (δ 171.7) due to the low-fielded chemical shifts. The remaining resonances of one sp^2 methine (δ 6.96 s; H-4), one oxygenated methine (δ 5.59 s; H-5) and a methoxyl group (δ 3.35 s; H-6), were put together based on the thoroughly HMBC correlation from C-2 (δ 171.7), C-4 (δ 140.9) and C-5 (δ 84.0) to H-4, H-5 and H-6. Two possible structures (**A** and **B**) were therefore proposed.

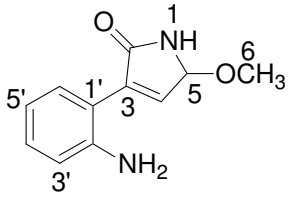


A



B

Due to the lack of further information, it is rather difficult to conclude with substantial evidence whether structures **A** or **B** is the right structure. However, it was believed that structure **B** could be the reasonable structure based on olefinic chemical shift, as the anisotropy from the phenyl ring could be the only possible influence that led to the low-fielded chemical shift of C-3. Compound **G9** (Fig. 27) was therefore proposed as a new amino phenyl pyrrolidone derivative closely related to compound **G4**.



G9

Figure 27. Proposed structures of compound **G9**

Table 13. ^{1}H and ^{13}C NMR spectral data of compound **G9** (500 MHz for ^{1}H; $CDCl_3$)

Position	δ_H (mult.; *J* in Hz)	δ_C (mult.)	HMBC correlation
1	6.22 (br s)	-	-
2	-	171.7 (C)	H-4
3	-	140.5 (C)	H-6′
4	6.96 (s)	140.9 (CH)	H-5
5	5.59 (s)	84.0 (CH)	H-4, H-6
6	3.35 (s)	52.3 (CH_3)	H-5
1′	-	116.9 (C)	H-3′
2′	-	145.4 (C)	H-4′, H-6′
3′	6.73 (d; 8.0)	117.1 (CH)	H-5′
4′	7.18 (dd; 7.0, 8.0)	130.5 (CH)	H-6′
5′	6.78 (dd; 7.0, 7.5)	118.7 (CH)	H-3′
6′	7.28 (m)	130.6 (CH)	H-4′, H-5′
2′-NH_2	4.36 (br s; 2H)	-	-

3.5 Cytotoxicity of isolated compounds

Cytotoxicity of compounds **G1-G4** were examined in the SRB assay against four different human cell lines (MCF-7, HT-29, KB and HeLa) (Skehan *et al*., 1990). The result is shown in Table 14. In this study, camptothecin was used as a positive control (IC_{50} against MCF-7 0.00057 mM). Compound **G3** showed the lowest IC_{50} value (0.00117 mM) against HeLa cell line whereas the IC_{50} values of this compound against other cell line were a magnitude higher. The new substance, compound **G4** [3-(*o*-aminophenyl)pyrrole], exhibited moderate inhibitory activity against all cancer cells except HeLa. Although *N*-(2-phenylethyl) acetamide, compound **G3**, showed only moderate cytotoxic activity in this study, it was reported to possess antifungal activity (Daoud *et al*., 1993) and weak phycotoxicity against the microalgae *Chlorella vulgaris*, *Chlorella salina* and *Scenedesmus subspicatus* (Shaaban *et al*., 2002). Two epimeric diketopiperzines cyclo(Pro-Phe), compounds **G1** and **G2**, were not cytotoxic against four human cell lines. However, other biological activities of these diketopiperzines were reported by other investigators including antifungal activity (Wang *et al*., 1999) and phytotoxin (Strobel *et al*., 1999). Compound **G2** was reported with strong antibacterial activity against *Vibrio anguillarum* with the MIC of 0.03-0.07 μg/ml (Fdhila *et al*., 2003). Strobel and colleagues (1991) suggested the importance of structure conformation in bioactivity and described the aromatic moiety and hydroxyl group as essential functional groups for activities. Moreover, their moderate fungicidic activity against *Cladosporium phlei* at a dose of 500 μg was reported by Seto *et al*. (2005). Cyclic dipeptides (diketopiperazines, DKPs) are a relatively unexplored class of bioactive peptides that may hold great promise for the future. Recently, the interest to these compounds is increased because of their significant bioactivity i.e. immunoreactivity (Wolf *et al*., 1994; Leduque *et al*., 1987), serum level elevating (Jara *et al*., 1997), and the role in cell-to-cell signalling mechanism in bacteria, so called ‘‘quorum sensing’’ (Klose, 2006; Rosa *et al*., 2003).

Table 14. Cytotoxicity of compounds **G1**, **G2**, **G3** and **G4**

Compound	IC_{50} (mM)			
	MCF-7	**KB**	**HT-29**	**HeLa**
G1	Not active	Not active	Not active	Not active
G2	Not active	Not active	Not active	Not active
G3	0.042	0.028	0.021	0.00117
G4	0.059	0.03	0.048	Not active
Camptothecin	0.00057	No test	No test	No test

CHAPTER 4

CONCLUSION

Twenty-seven strains of marine gliding bacteria were isolated from the specimens collected from coastlines in the Gulf of Thailand. It was found that biofilms provided the highest number of isolates followed by sponges, plant materials, algae and crab carcass. Subculturing and micromanipulation techniques were the most appropriate purification and isolation techniques for gliding bacteria in this study. Based on 16S rRNA gene sequence and phylogenetic analyse, the representative isolates were identified and classified to six different groups within the *Cytophaga-Flavobacterium-Bacteriodes* (CFB) group. Because of their similarities in 16S rRNA gene sequences, Groups 1, 4, 5 and 6 were proposed as novel genera including *Rapidithrix thailandica* gen. nov., sp. nov. (Gr. 4), *Aureispira marina* gen. nov., sp. nov. (Gr. 5) and *Aureispira maritima* sp. nov. (Gr. 6). Group 2 was identified as *Tenacibaculum mesophilum* whereas group 3 could potentially be a new species within *Fulvivirga kasyanovii*. During the course of cytotoxcity screening, forty-two marine gliding bacterial isolates were cultivated in four cultivation media (Vy/2, RL 1, CY and SK) and were screened for cytotoxicity against four human cell lines including cervical cancer (HeLa), colon cancer (HT-29), oral cancer (KB) and breast adenocarcinoma (MCF-7). The results showed that the members of group 2 and 4 were capable of producing metabolites with cytotoxicities against all cell lines whereas the members of group 6 and the unidentified group could produce crude extracts with cytotoxicites against only HeLa and HT-29. Interestingly, groups 1, 3 and 5 could not produce any crude extracts with cytotoxicity. The CY medium was shown to be the most suitable medium for cytotoxic metabolites production yielding 23 cytotoxic extracts, followed by SK (11 extracts), RL 1 (8 extracts) and Vy/2 (7 extracts). Moreover, CY medium was the only medium which gave the active crude extract against MCF-7. Three extracts obtained from *Rapidithrix thailandica* (TISTR1768), *Rapidithrix thailandica* (TISTR1741) and *Tenacibaculum mesophilum* (TISTR1782) were submitted to the bioassay guided fractionation in order to isolate and determine the compounds present in the

extract. Nine metabolites including cyclo(L-Pro-D-Phe) [**G1**], cyclo(L-Pro-L-Phe) [**G2**], *N*-(2-phenylethyl) acetamide [**G3**], cyclo(L-Pro-D-Tyr) [**G5**], cyclo(L-Pro-L-Tyr) [**G6**], cyclo(8-hydroxy-L-Pro-L-Leu) [**G7**], cyclo(8-hydroxy-L-Pro-D-Phe) [**G8**] and two novel compounds, 3-(2'-aminophenyl)-pyrrole [**G4**] and 3-(2-Amino-phenyl)-5-methoxy-1,5-dihydro-pyrrol-2-one [**G9**], were isolated. Based on IC_{50} values *N*-(2-phenylethyl) acetamide [**G3**] and 3-(2'-aminophenyl)-pyrrole [**G4**] were considered moderately active against all four cell lines and three cell lines (HT-29, KB and MCF-7), respectively, whereas cyclo(L-Pro-D-Phe) [**G1**] and cyclo(L-Pro-L-Phe) [**G2**] exhibited no cytotoxicity.

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APPENDIX A

16S rRNA gene sequences of marine gliding bacterial isolates

1) GB058 (TISTR1761)

ACGCTATTTCAAGCCTAACACNTGCAAGTCGAGGGGTATATAGTAGCTTGCTACTATA
GAGACCGGCGCACGGGTGAGTACCGCGTATGCAATCTACCTTTTGCTGGGGGATAGC
CCGAAGAAATTTGGATTAATACCCCATGGTATACAGTTTTGGCATCGGAACTGTATTA
AAGCTGAGGCGGCAAAAGATGAGCATGCGTTCCATTAGCTGGTTGGTATGGTAACGG
CATACCAAGGCTACGATGGATCGGGGTCCTGAGAGGGAGATCCCCCACACTGGAACT
GAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATATTGGACAATGGGC
GGGAGCCTGATCCAGCCATGCCGCGTGCAGGAAGACTGCCCTATGGGTTGTAAACTG
CTTTTCTACGAGAAGAATAAGGGCTACGTGTAGCTTGATGACGGTATCGTAGGAATA
AGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTATC
CGGAATCATTGGGTTTAAAGGGTCCGTAGGCGGATGGATAAGTCAGTGGTGAAAGTT
TGCCGCTTAACGGTAAAATTGCCTCTGATACTGTCTATCTTGAATGTGTGTGAAGTGA
TTAGAATATGTAGTGTAGCGGTGAAATGCATAGATATTACATAGAATACCGATTGCG
AAGGCAGGTCACTAACACATGATTGACGCTGATGGACGAAAGCGTGGGTAGCGAACA
GGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGATACTAGCTGTTGGGGTGT
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GAATGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTA
ATTCGATGATACGCGAGGAACCTTACCAAGGCTTAAATGTATGTTGACGTAATTGGA
AACAGTTATTTCTTCGGACAATATACAAGGTGCTGCATGGTTGTCGTCAGCTCGTGCC
GTGAGGTGTCAGGTTAAGTCCTATAACGAGCGCAACCCCTGTGGTTAGTTACCAGCAT
GTAATGGTGGGGACTCTAACCAGACTGCCGGTGCAAACCGTGAGGAAGGTGGGGATG
ACGTCAAATCATCACGGCCCTTACGTCTTGGGCTACACACGTGCTACAATGGTCGGTA
CAGCGAGCAGCCACTGCGTGAGCAGGCGCGAATCTATAAAACCGATCACAGTTCGGA
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CCGGGGGTACCTGAAGTCGGTGACCGCAAGGAGCTGCCTAGGGTAAAACTGGTAACT
GGGGCTAAGTCGTAACAAGGTAACCGAA

2) GB060 (TISTR1763)

TCCAGCCTAACACATGCAAGTCGAGGGGTATATAGTAGCTTGCTACTATAGAGACCG
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AATTTGGATTAATACCCCATGGTATACAGTTTTGGCATCGGAACTGTATTAAAGCTGA
GGCGGCAAAAGATGAGCATGCGTTCCATTAGCTGGTTGGTATGGTAACGGCATACCA
AGGCTACGATGGATCGGGGTCCTGAGAGGGAGATCCCCCACACTGGAACTGAGACAC
GGACCAGACTCCTACGGGAGGCAGCAGTGAGGAATATTGGACAATGGGCGGGAGCC
TGATCCAGCCATGCCGCGTGCAGGAAGACTGCCCTATGGGTTGTAAACTGCTTTTCTA
CGAGAAGAATAAGGGCTACGTGTAGCTTGATGACGGTATCGTAGGAATAAGCACCGG
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TTGGGTTTAAAGGGTCCGTAGGCGGATGGATAAGTCAGTGGTGAAAGTTTGCCGCTT
AACGGTAAAATTGCCTCTGATACTGTCTATCTTGAATGTGTGTGAAGTGATTAGAATA
TGTAGTGTAGCGGTGAAATGCATAGATATTACATAGAATACCGATTGCGAAGGCAGG
TCACTAACACATGATTGACGCTGATGGACGAAAGCGTGGGTAGCGAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGATGGATACTAGCTGTTGGGGTGTATGTCTCAG
TGGTTAAGCGAAAGTGATAAGTATCCCACCTGGGGAGTACGTTCGCAAGAATGAAAC
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TACGCGAGGAACCTTACCAAGGCTTAAATGTATGTTGACGTAATTGGAAACAGTTATT
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GCAACTCGACTCCGTGAAGCTGGAATCGCTAGTAATCGGATATCAGCCATGATCCGG
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CCTGAAGTCGGTGACCGCAAGGAGCTGCCTAGGGTAAAACTGGTAACTGGGGCTAAG
TCGTAACAAGGTAACCGA

3) TISTR1755 (AB265190)

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TGGCATCATTTTTAAATTAAAGATTTATTGGTACAAGATGACTATGCGTCCTATTAGC
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ATCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGG
AATATTGGTCAATGGAGGCAACTCTGAACCAGCCATGCCGCGTGCAGGAAGACTGCC
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CGGTACTGTAAGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA
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4) GB074 (TISTR1778 = AB265188)

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GGCATCATTTTTAAATTAAAGATTTATTGGTACAAGATGACTATGCGTCCTATTAGCT
AGATGGTAAGGTAACGGCTTACCATGGCAACGATAGGTAGGGGGTCTGAGAGGATTA
TCCCCCACACTGGTACTGAGACACGGACCAGACTCCTACGGGAGGCAGCAGTGAGGA
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TATGGGTTGTAAACTGCTTTTATACAGGAAGAAACAGAACTACGTGTAGTTCCTTGAC
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CAAGCCATGGAAGCTGGGGGTGCCTGAAAGTCGGTTACCGCAAGGAGCTGCCTAGGG
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5) GB078 (TISTR1782 = AB265189)

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GGCATCATTTTTAAATTAAAGATTTATTGGTACAAGATGACTATGCGTCCTATTAGCT
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CGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGACAC
TAGTTGTTGGGATTTGTCTCAGTGACTAAGCGAAAGTGATAAGTGTCCCACCTGGGGA
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AGCATGTGGTTTAATTCGATGATACGCGAGGAACCTTACCAGGGCTTAAATGTAGAG
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GAAGGTGGGGATGACGTCAAATCATCACGGCCCTTACGTCCTGGGCTACACACGTGC
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TATCACAGTTCGGATCGGAGTCTGCAACTCGACTCCGTGAAGCTGGAATCGCTAGTAA
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6) GB080 (TISTR1784)

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TACAAGATGACTATGCGTCCTATTAGCTAGATGGTAAGGTAACGGCTTACCATGGCA
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CGTTCCCGGGCCTTGTACACACCGCCCGTCAAGCCATGGAAGCTGGGGGTGCCTGAA

GTCGGTTACCGCAAGGAGCTGCCTAGGGTAAAACTGGTAACTAGGGCTAAGTCGTAA
CAAGGTAACCGAT

7) TISTR1692

TCGGCTTAATACATGCAAGTCGAACGGTAACAGCAACTTCGGTTGGCTGACGAGTGG
CGCACGGGTGAGTAACGCGTACAAAACCTACCTTTTACTGGGGGAAAGTCGCTGGAA
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CGGTCGGTAAAAGATGGTTGTGCGTCCCATTAGCTAGATGGTAAGGTAACGGCTTAC
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8) TISTR1726 (AB278130)

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9) TISTR1726 (AB278130)

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9) AB265191

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10) TISTR1721

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CGTGTGTGATGAAGGCCCTTTGGGTTGTAAAGCACTTTTGTCTGGGAAGAATGGCTTT
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GTAACCGAA

11) TISTR1719 (AB245933)

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ggtaccagaagaataagcaccggctaactccgtgccagcagccgcggtaatacggagggtgcaagcgttatccggaatcactgggtttaaa
gggtacgtaggcggcggtataagtcagacgttaaagactggggctaaaccctggtacgcgtttgaaactgtacggcttgaatctgttcgaggt
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gtacgaaagcgtgggtagcgaacaggattagataccctggtagtccacgccctaaacgatgctaactggttatttatcggaataggtaagtaac
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12) TISTR1728 (AB245934)

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13) TISTR1731 (AB245935)

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14) TISTR1741 (AB265183)

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ATCTGGATTAATACCCCATAACACAGGGGTTCCGCATGGGACTATTGGTTAAAGCTTC
GGCGGTTGTGGATGAGCATGCGTCCCATTAGCTAGTAGGCGAGGTAACGGCTCACCT
AGGCGATGATGGGTAGGGGTTCTGAGAGGAAGGTCCCCCACACTGGTACTGAGATAC
GGACCAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGGGCAATGGGCGCAAGNC
TGACCCAGCCATGCCGCGTGCAGGAAGGCCCTCGGGTTGTAAACTGCTTTTATACGGG
AAGAACTCCACTATGCGTAGTGGTTTGACGGTACCGTACGAATAAGCACCGGCTAAC
TCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTGTCCGGATTTATTGGG
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AAGCGTGCCATTGATACTGTCGAACTTGAGTCCTGTAGAGGTAGGCGGAATTCCGCAT
GTAGCGGTGAAATGCATAGATATGCGGAGGAACTCCAAAAGCGAAGGCAGCTTACTG
GACAGGAACTGACGCTGAGGTACGAAAGCGTGGGGAGCGAACAGGATTAGATACCC
TGGTAGTCCACGCCGTAAACGATGGTTACTAGGTGTGCGCGACATACAGTGCGTGCC
CAAGCGAAAGCGATAAGTAACCCACCTGGGGAGTACGCTCGCAAGAGTGAAACTCA
AAGGAATTGACGGGGGTCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGATAC
GCGAGGAACCTTACCTGGGCTAGAATGCGAGTGACGGATCTAGAGATAGATCTTCCT
TCGGGCACAAAGCAAGGTGCTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGTGTTGG
GTTAAGTCCCGCAACGAGCGCAACCCCTACCCTTAGTTGCCATCAGATAATGCTGGGG
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TCATGGCCCTTACGCCCAGGGCTACACACGTGCTACAATGGCGCATACAGAGGGTAG
CTACCTGGCAACAGGATGCGAATCTCAAAAAGTGCGTCTCAGTTCGGATTGTGGTCTG
CAACTCGACCACATGAAGTTGGAATCGCTAGTAATCGCGCATCAGCAATGGCGCGGT
GAATACGTTCCCGGACCTTGTACACACCGCCCGTCAAGCCATGGGAGTTGGGTGGAC
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TCGTAACAAGGTAACCGAAATT

15) TISTR1742

GCTCCAGGATGAACGCTAGCGGCAGGCCTAATACATGCAAGTCGAACGGCAGCACGA
TCGCTTCGGTGATTGGTGGCGAGTGGCGCACGGGTGCGTAACGCGTATGCAATCTGN
CCACAACAGGGGGATAGCCCAGAGAAATCTGGATTAATACCCCATAACACAGGGGTT
CCGCATGGGACTATTGGTTAAAGCTTCGGCGGTTGTGGATGAGCATGCGTCCCATTAG
CTAGTAGGCGAGGTAACGGCTCACCTAGGCGATGATGGGTAGGGGTTCTGAGAGGAA
GGTCCCCCACACTGGTACTGAGATACGGACCAGACTCCTACGGGAGGCAGCAGTAGG
GAATATTGGGCAATGGGCGCAAGCCTGACCCAGCCATGCCGCGTGCAGGAAGACGGC
CCTCGGGTTGTAAACTGCTTTTATACGGGAAGAACTCCACTATGCGTAGTGGTTTGAC
GGTACCGTACGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG
GGTGCAAGCGTTGTCCGGATTTATTGGGTTTAAAGGGTACGTAGGCGGTTCGGTAAGT
CAGTGGTGAAATTTTACAGCTCAACTGTAAGCGTGCCATTGATACTGTCGAACTTGAG
TCCTGTAGAGGTAGGCGGAATTCCGCATGTAGCGGTGAAATGCATAGATATGCGGAG
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CGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGTTAC
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AGTGACGGATCTAGAGATAGATCTTCCTTCGGGCACAAAGCAAGGTGCTGCATGGCT
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GAGGAAGGAGGGGACGATGTCAAGTCATCATGGCCCTTACGCCCAGGGCTACACACG
TGCTACAATGGCGCATACAGAGGGTAGCTACCTGGCAACAGGATGCGAATCTCAAAA
AGTGCGTCTCAGTTCGGATTGTGGTCTGCAACTCGACCACATGAAGTTGGAATCGCTA
GTAATCGCGCATCAGCAATGGCGCGGTGAATACGTTCCCGGACCTTGTACACACCGC
CCGTCAAGCCATGGGAGTTGGGTGGACCTGAAGATTGTGACCATTTAGGAGCAGTTT
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16) TISTR1747 (AB265184)

CAAGCTCAGGATGAACGCTAGCGGCAGGCCTAATACATGCAAGTCGAACGGCAGCAC
GATCGCTTCGGTGATTGGTGGCGAGTGGCGCACGGGTGCGTAACGCGTATGCAATCT
GCCCACAACAGGGGGATAGCCCAGAGAAATCTGGATTAATACCCCATAACACAGGGG
TTCCGCATGGGACTATTGGTTAAAGCTTCGGCGGTTGTGGATGAGCATGCGTCCCATT
AGCTAGTAGGCGAGGTAACGGCTCACCTAGGCGATGATGGGTAGGGGTTCTGAGAGG
AAGGTCCCCCACACTGGTACTGAGATACGGACCAGACTCCTACGGGAGGCAGCAGTA
GGGAATATTGGGCAATGGGCGCAAGCCTGACCCAGCCATGCCGCGTGCAGGAAAGAC
GGCCCTCGGGTTGTAAACTGCTTTTATACGGGAAGAACTCCACTATGCGTAGTGGTTT
GACGGTACCGTACGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACG
GAGGGTGCAAGCGTTGTCCGGATTTATTGGGTTTAAAGGGTACGTAGGCGGTTCGGT
AAGTCAGTGGTGAAATTTTACAGCTCAACTGTAAGCGTGCCATTGATACTGTCGAACT
TGAGTCCTGTAGAGGTAGGCGGAATTCCGCATGTAGCGGTGAAATGCATAGATATGC
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GCGGTGGAGCATGTGGTTTAATTCGATGATACGCGAGGAACCTTACCTGGGCTAGAA
TGCGAGTGACGGATCTAGAGATAGATCTTCCTTCGGGCACAAAGCAAGGTGCTGCAT
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CCGCCCGTCAAGCCATGGGAGTTGGGTGGACCTGGAAGATTGTGACCATTTAGGAGC
AGTTTAGGGTCAAACCAGCGACTGGGGCTAAGTCGTAACAAGGTAACCGA

17) TISTR1749 (AB265185)

TTGAGTTTGATTCCAAGCTCAGGATGAACGCTAGCGGCAGGCCTAATACATGCAAGT
CGAACGGCACATGCAAGTCGAACGGCAGCACGATCGCTTCGGTGATTGGTGGCGAGT
GGCGCACGGGTGCGTAACGCGTATGCAATCTGCCCACAACAGGGGGATAGCCCAGAG
AAATCTGGATTAATACCCCATAACACAGGGGTTCCGCATGGGACTATTGGTTAAAGCT
TCGGCGGTTGTGGATGAGCATGCGTCCCATTAGCTAGTAGGCGAGGTAACGGCTCAC
CTAGGCGATGATGGGTAGGGGTTCTGAGAGGAAGGTCCCCCACACTGGTACTGAGAT
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CCTGACCCAGCCATGCCGCGTGCAGGAAGACGGCCCTCGGGTTGTAAACTGCTTTTAT
ACGGGAAGAACTCCACTATGCGTAGTGGTTTGACGGTACCGTACGAATAAGCACCGG
CTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTGTCCGGATTTA
TTGGGGTTTAAAGGGTACGTAGGCGGTTCGGTAAGTCAGTGGTGAAATTTTACAGCTC
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CTTACTGGACAGGAACTGACGCTGAGGTACGAAAGCGTGGGGAGCGAACAGGATTA
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GCGTGCCCAAGCGAAAGCGATAAGTAACCCACCTGGGGAGTACGCTCGCAAGAGTGA
AACTCAAAGGAATTGACGGGGGTCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGA
TGATACGCGAGGAACCTTACCTGGGCTAGAATGCGAGTGACGGATCTAGAGATAGAT
CTTCCTTCGGGCACAAAGCAAGGTGCTGCATGGCTGTCGTCAGCTCGTGCCGTGAGGT
GTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTACCCTTAGTTGCCATCAGATAATGC
TGGGGACTCTAAGGGGACTGCCTACGCAAGTAGAGAGGAAGGAGGGGACGATGTCA
AGTCATCATGGCCCTTACGCCCAGGGCTACACACGTGCTACAATGGCGCATACAGAG
GGTAGCTACCTGGCAACAGGATGCGAATCTCAAAAAGTGCGTCTCAGTTCGGATTGT
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CGCGGTGAATACGTTCCCGGACCTTGTACACACCGCCCGTCAAGCCATGGGAGTTGG
GTGGACCTGAAGATTGTGACCATTTAGGAGCAGTTTAGGGTCAAACCAGCGACTGGG
GCTAAGTCGTAACAAGGTAACCGA

18) TISTR1750 (AB265192)

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TCTGGATTAATACCCCATAACACAGGGGTTCCGCATGGGACTATTGGTTAAAGCTTCG
GCGGTTGTGGATGAGCATGCGTCCCATTAGCTAGTAGGCGAGGTAACGGCTCACCTA
GGCGATGATGGGTAGGGGTTCTGAGAGGAAGGTCCCCCACACTGGTACTGAGATACG
GACCAGACTCCTACGGGAGGCAGCAGTAGGGAATATTGGGCAATGGGCGCAAGCCTG
ACCCAGCCATGCCGCGTGCAGGAAGACGGCCCTCGGGTTGTAAACTGCTTTTATACCC
TCGGGTTGTAAACTGCTTTTATACGGGAAGAACTCCACTATGCGTAGTGGTTTGACGG
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GTGGTGAAATTTTACAGCTCAACTGTAAGCGTGCCATTGATACTGTCGAACTTGAGTC
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GTGTGCGCGACATACAGTGCGTGCCCAAGCGAAAGCGATAAGTAACCCACCTGGGGA
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AGCATGTGGTTTAATTCGATGATACGCGAGGAACCTTACCTGGGCTAGAATGCGAGT
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GTCAGCTCGTGCCGTGAGGTGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTACCCT
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TACAATGGCGCATACAGAGGGTAGCTACCTGGCAACAGGATGCGAATCTCAAAAAGT
GCGTCTCAGTTCGGATTGTGGTCTGCAACTCGACCACATGAAGTTGGAATCGCTAGTA
ATCGCGCATCAGCAATGGCGCGGTGAATACGTTCCCGGACCTTGTACACACCGCCCGT
CAAGCCATGGGAGTTGGGTGGACCTGAAGATTGTGACCATTTAGGAGCAGTTTAGGG
TCAAACCAGCGACTGGGGCTAAGTCGTAACAAG

19) TISTR1710

CTCCAGGATGAACGCTAGCGGCAGGCCTAATACATGCAAGTCGAACGGCAGCACGAT
CGCTTCGGTGATTGGTGGCGAGTGGCGCACGGGTGCGTAACGCGTAGGCAATCTGCC
CACAACAGGGGGATAGCCCAGAGAAATCTGGATTAATACCCCATAACACAGGGGTTC
CGCATGGGACTATTGGTTAAAGCTTCGGCGGTTGTGGATGAGCATGCGTCCCATTAGC
TAGTAGGCGAGGTAACGGCTCACCTAGGCGATGATGGGTAGGGGTTCTGAGAGGAAG
GTCCCCCCACACTGGTACTGAGATACGGACCAGACTCCTACGGGAGGCAGCAGTAGGG
AATATTGGGCAATGGGCGCAAGCCTGACCCAGCCATGCCGCGTGCAGGAAGACGGCC
CTCGGGTTGTAAACTGCTTTTATACGGGAAGAACTCCACTATGCGTAGTGGTTTGACG
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GTGCAAGCGTTGTCCGGATTTATTGGGTTTAAAGGGTACGTAGGCGGTTCGGTAAGTC
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GTGCGTCTCAGTTCGGATTGTGGTCTGCAACTCGACCACATGAAGTTGGAATCGCTAG
TAATCGCGCATCAGCAATGGCGCGGTGAATACGTTCCCGGACCTTGTACACACCGCCC
GTCAAGCCATGGGAGTTGGGTGGACCTGAAGATTGTGACCATTTAGGAGCAGTTTAG
GGTCAAACCAGCGACTGGGGCTAAGTCGTAACAAGGTAACCGA

20) GB057 (TISTR1760 = AB265186)

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GCCCACAACAGGGGGATAGCCCAGAGAAATCTGGATTAATACCCCATAACACAGGGG
TTCCGCATGGGACTATTGGTTAAAGCTTCGGCGGTTGTGGATGAGCATGCGTCCCATT
AGCTAGTAGGCGAGGTAACGGCTCACCTAGGCGATGAATGGGTAGGGGTTCTGAGAG
GAAGGTCCCCCACACTGGTACTGAGATACGGACCAGACTCCTACGGGAAGGCAGCAG
TAGGGAATATTGGGCAATGGGCGCAAGCCTGACCCAGCCATGCCGCGTGCAGGAAAG
ACGGCCCTCGGGTTGTAAACTGCTTTTATACGGGAAGAACTCCACTATGCGTAGTGGT
TTGACGGTACCGTACGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATA
CGGAGGGTGCAAGCGTTGTCCGGATTTATTGGGTTTAAAGGGTACGTAGGCGGTTCG
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CTTGAGTCCTGTAGAGGTAGGCGGAATTCCGCATGTAGCGGTGAAATGCATAGATAT
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AAGCGGTGGAGCATGTGGTTTAATTCGATGATACGCGAGGAACCTTACCTGGGCTAG
AATGCGAGTGACGGATCTAGAGATAGATCTTCCTTCGGGCACAAAGCAAGGTGCTGC
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AGTAGAGAGGAAGGAGGGGACGATGTCAAGTCATCATGGCCCTTACGCCCAGGGCTA
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TCGCTAGTAATCGCGCATCAGCAATGGCGCGGTGAATACGTTCCCGGACCTTGTACAC
ACCGCCCGTCAAGCCATGGGAGTTGGGTGGACCTGAAAGATTGTGACCATTTAGGAG
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21) GB062 (TISTR1766)

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ATGGGACTATTGGTTAAAGCTTCGGCGGTTGTGGATGAGCATGCGTCCCATTAGCTAG
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CCCCACACTGGTACTGAGATACGGACCAGACTCCTACGGGAGGCAGCAGTAGGGAAT
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GGGTTGTAAACTGCTTTTATACGGGAAGAACTCCACTATGCGTAGTGGTTTGACGGTA
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GCATGTGGTTTAATTCGATGATACGCGAGGAACCTTACCTGGGCTAGAATGCGAGTG
ACGGATCTAGAGATAGATCTTCCTTCGGGCACAAAGCAAGGTGCTGCATGGCTGTCG
TCAGCTCGTGCCGTGAGGTGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTACCCTT
AGTTGCCATCAGATAATGCTGGGGACTCTAAGGGGACTGCCTACGCAAGTAGAGAGG
AAGGAGGGGACGATGTCAAGTCATCATGGCCCTTACGCCCAGGGCTACACACGTGCT
ACAATGGCGCATACAGAGGGTAGCTACCTGGCAACAGGATGCGAATCTCAAAAAGTG
CGTCTCAGTTCGGATTGTGGTCTGCAACTCGACCACATGAAGTTGGAATCGCTAGTAA
TCGCGCATCAGCAATGGCGCGGTGAATACGTTCCCGGACCTTGTACACACCGCCCGTC
AAGCCATGGGAGTTGGGTGGACCTGAAGATTGTGACCATTTAGGAGCAGTTTAGGGT
CAAACCAGCGACTGGGGCTAAGTCGTAACAAGGTAACCGAA

22) GB064 (TISTR1768)

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23) GB068 (TISTR1772 = AB265187)

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24) GB084 (TISTR1765)

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25) TISTR1736 (AB284517)

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26) GB076 (TISTR1780)

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27) GB077 (TISTR1781)

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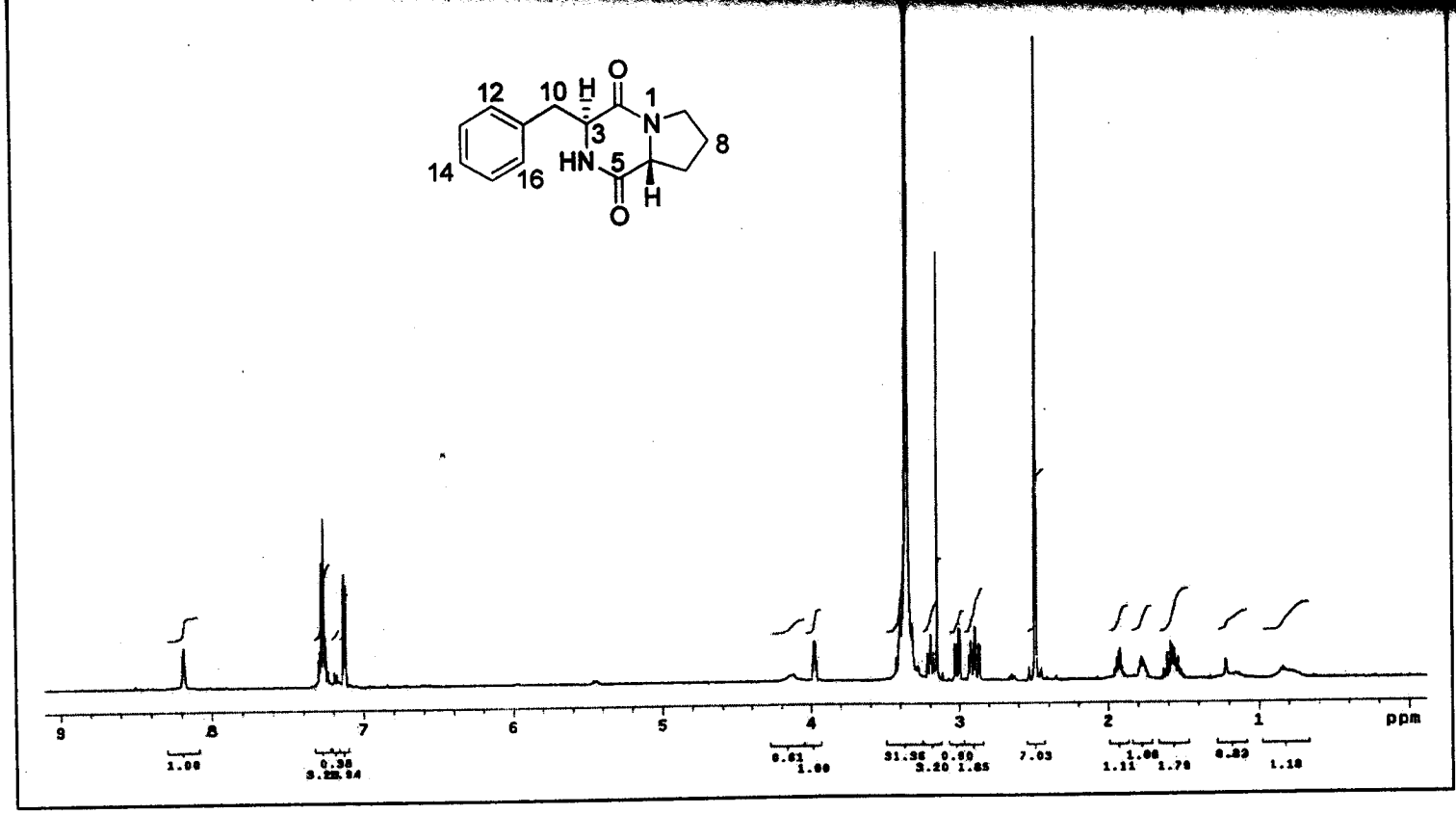
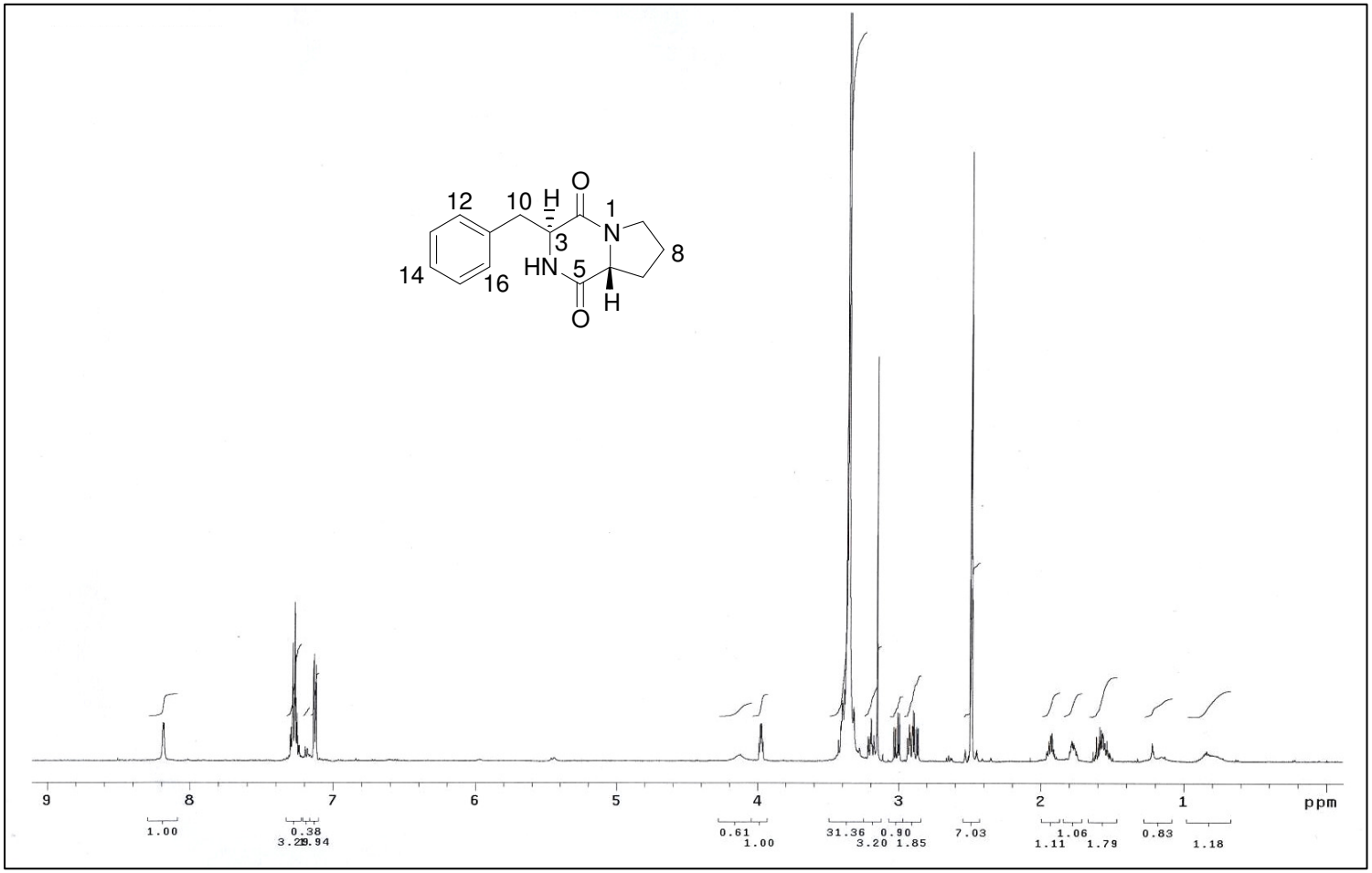
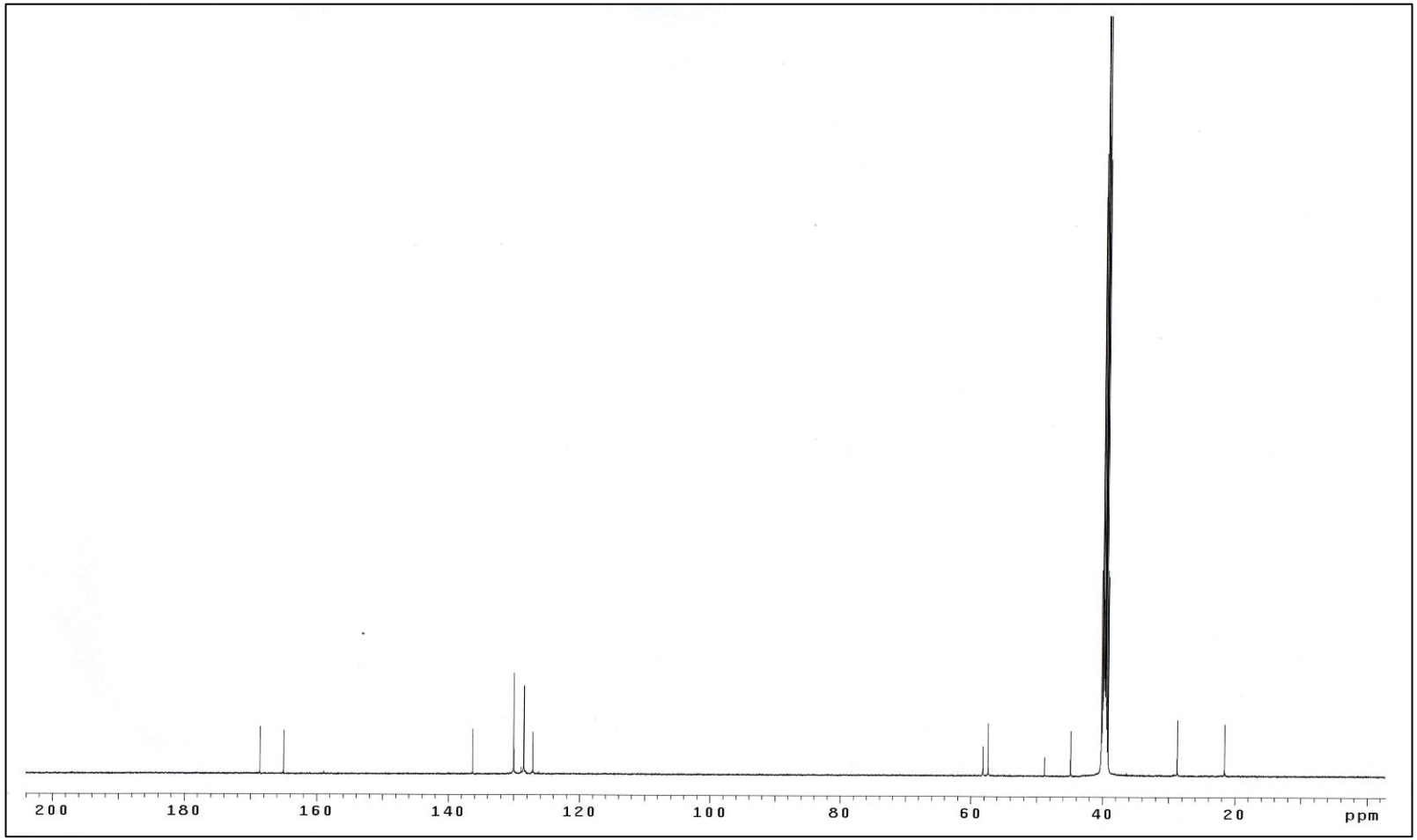


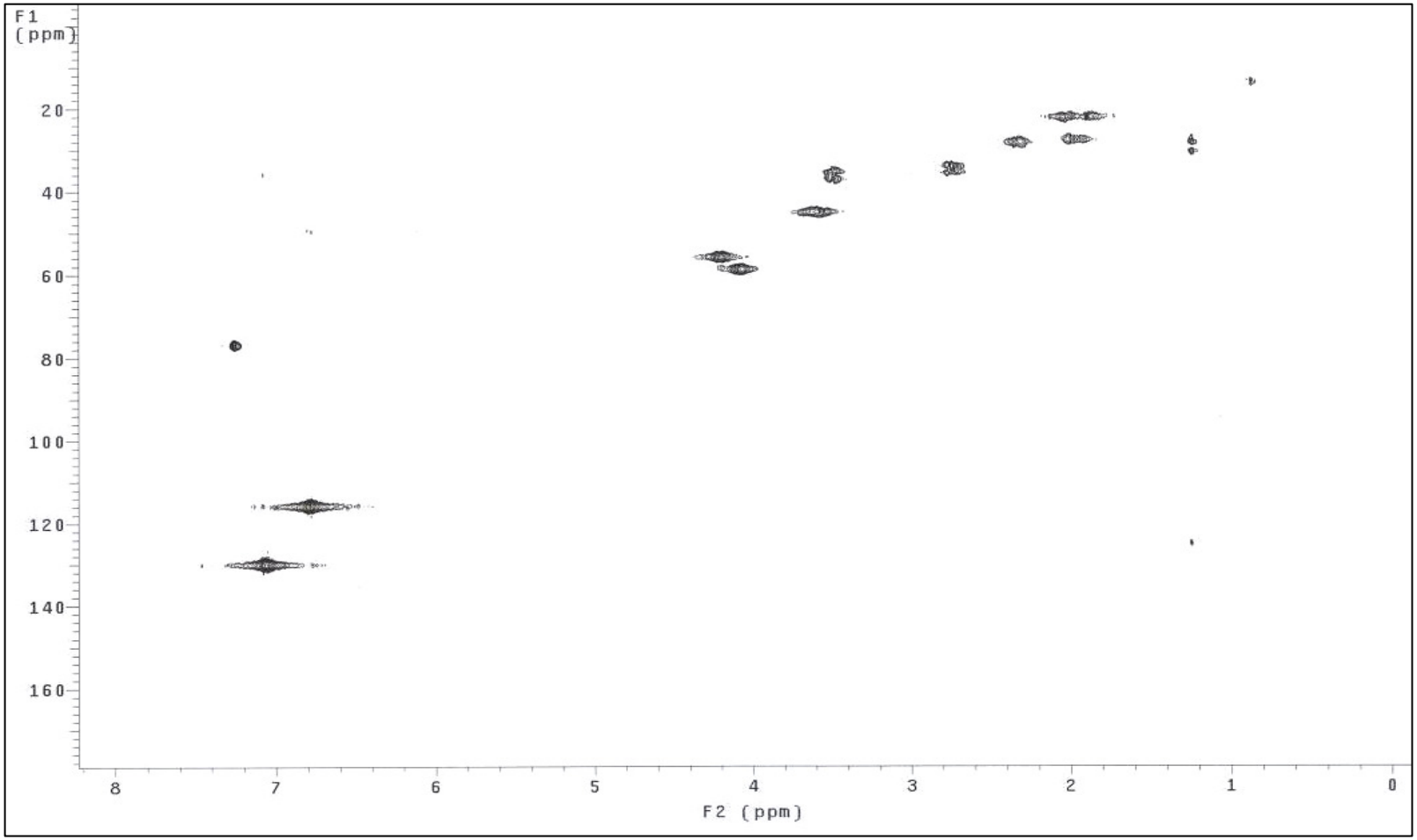
^{1}H NMR spectrum of compound **G1** (500 MHz, DMSO-d_6)



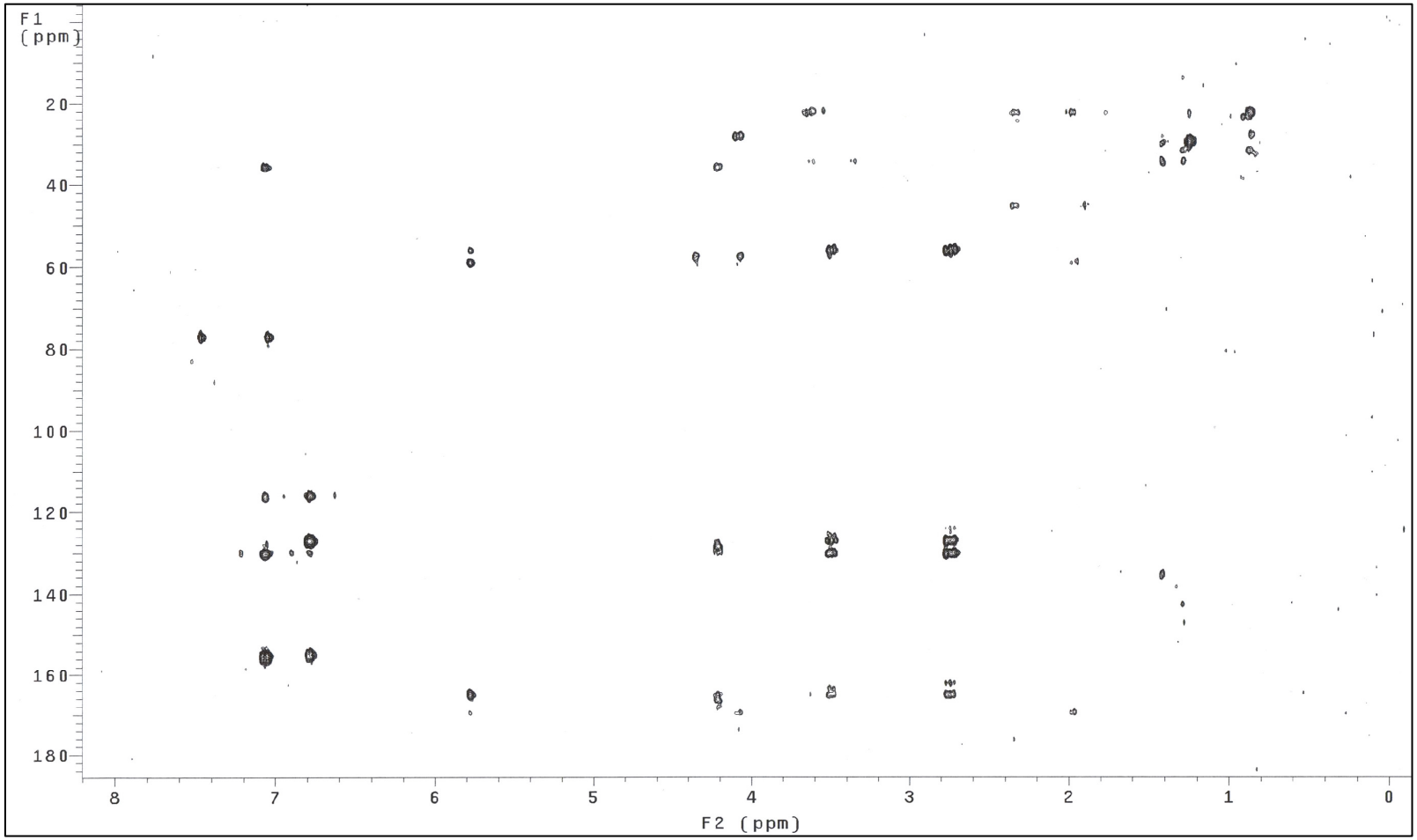
^{1}H NMR spectrum of compound **G1** (500 MHz, DMSO-d_6)



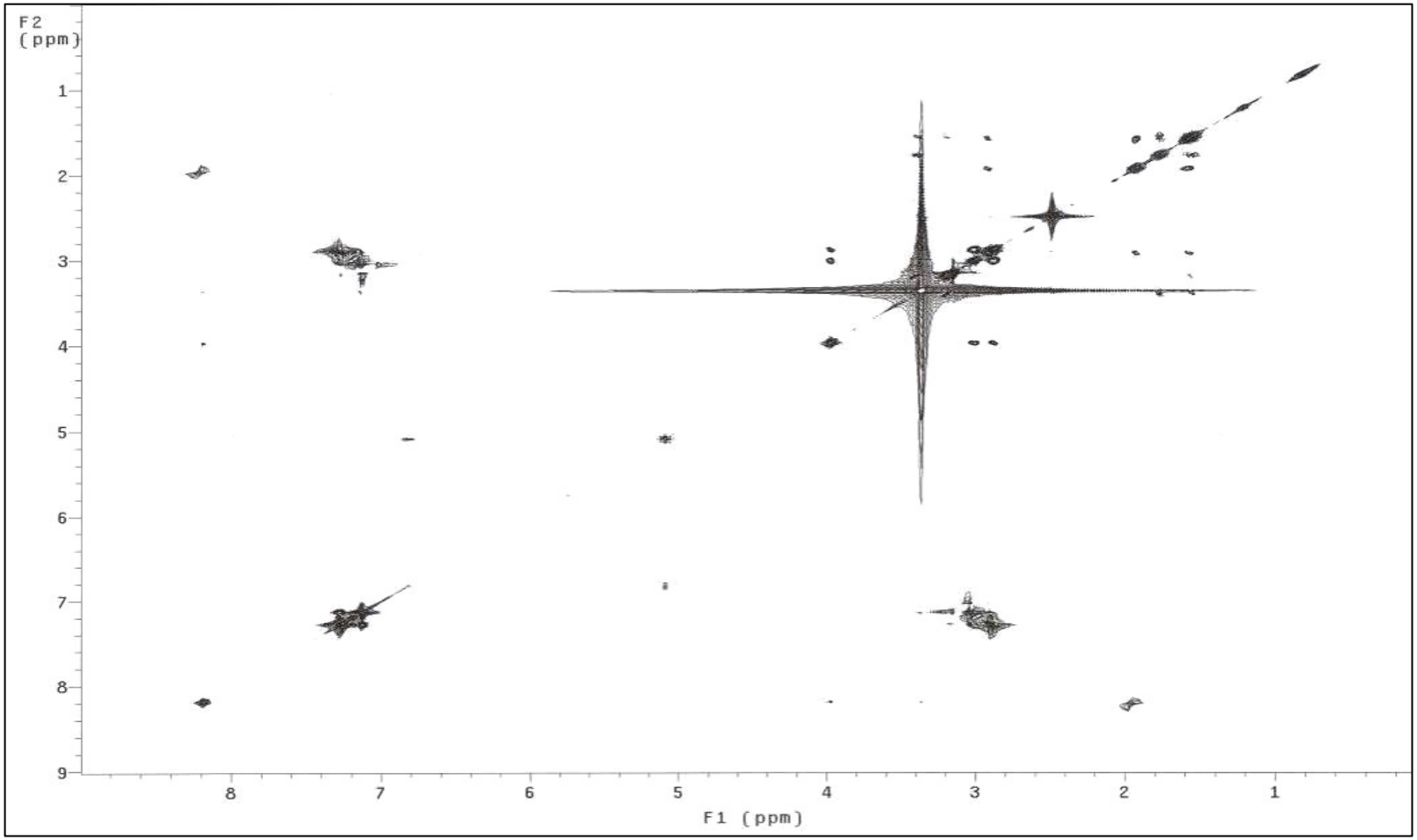
^{13}C NMR spectrum of compound **G1** (300 MHz, DMSO-d_6)



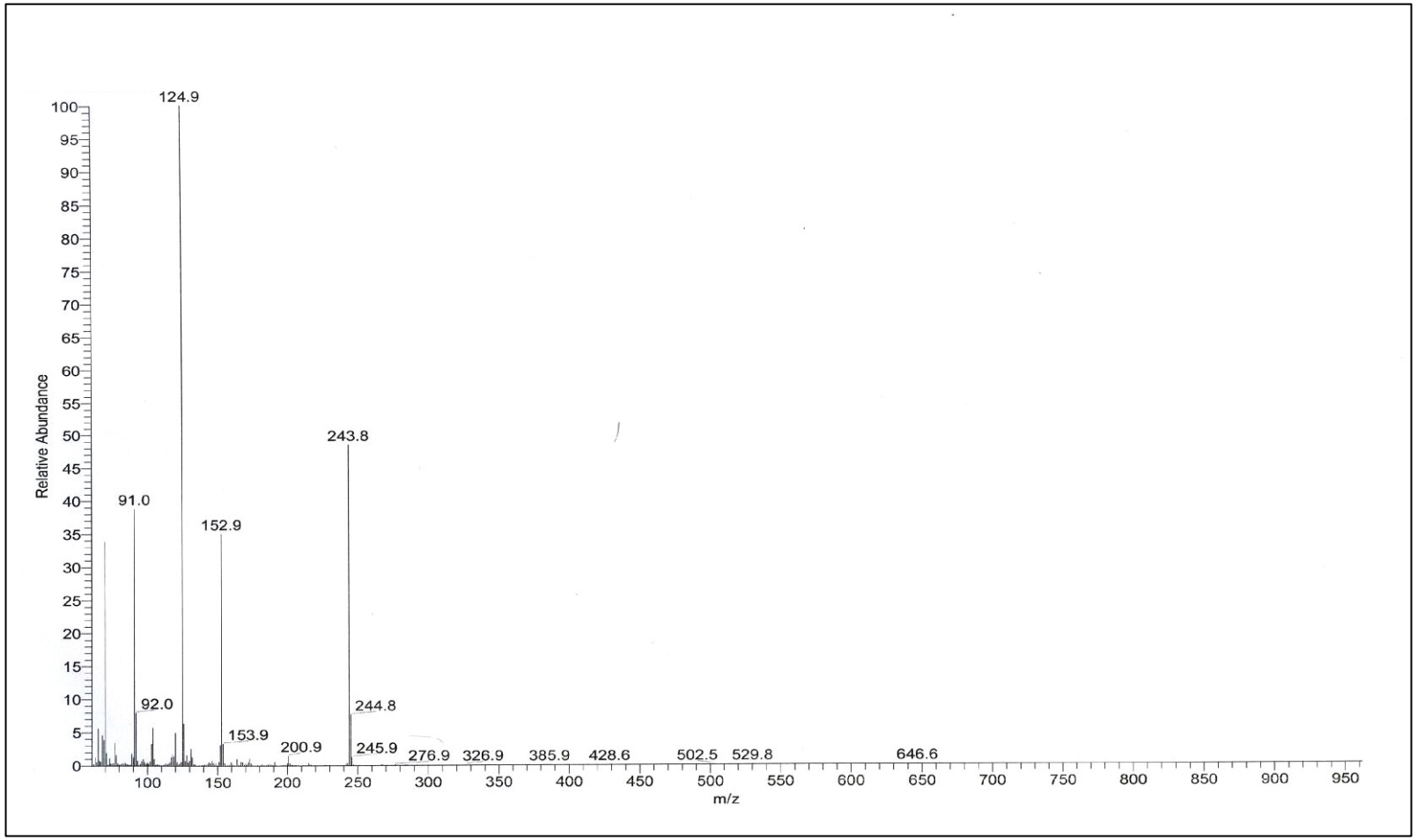
HMQC spectrum of compound **G1**



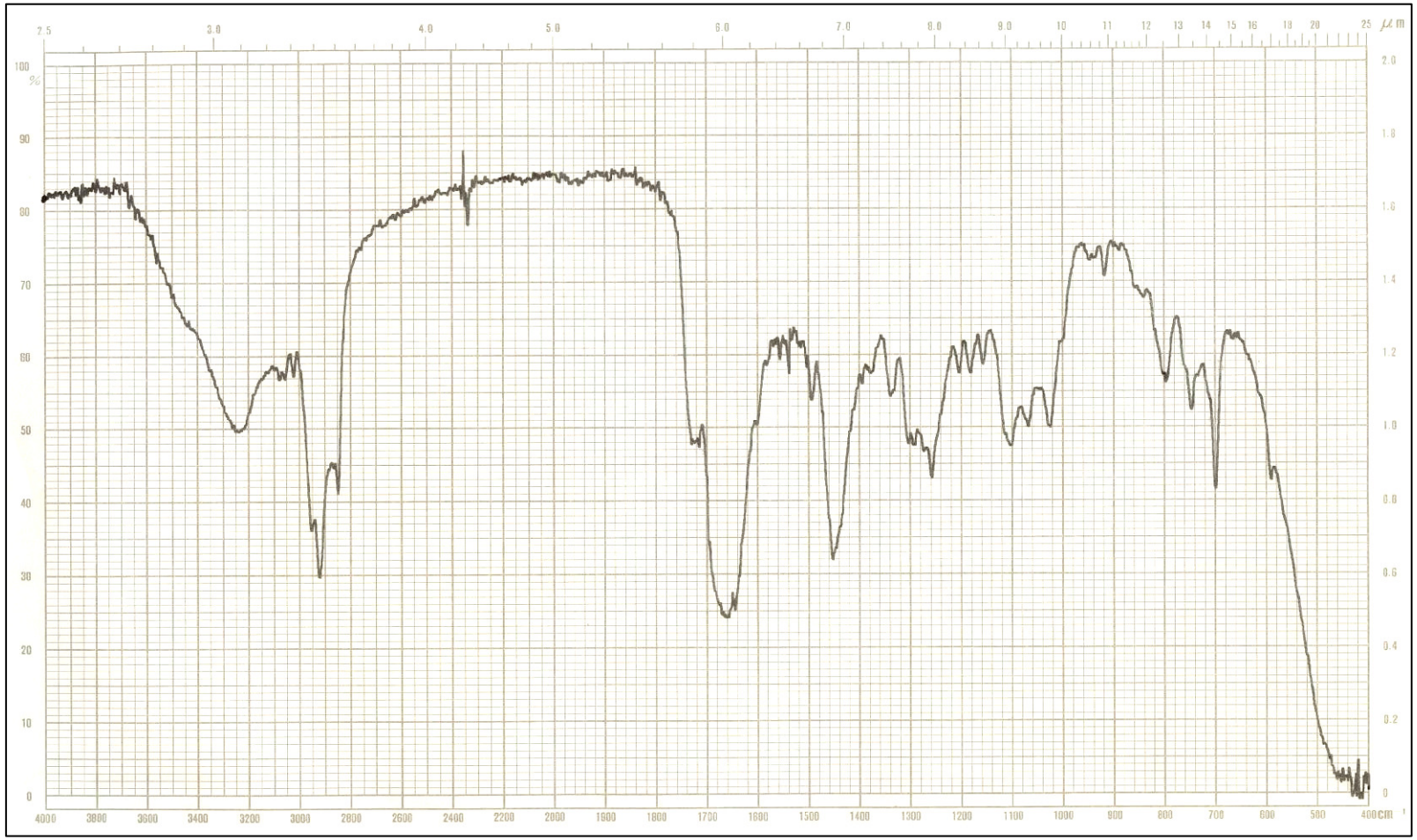
HMBC spectrum of compound **G1**



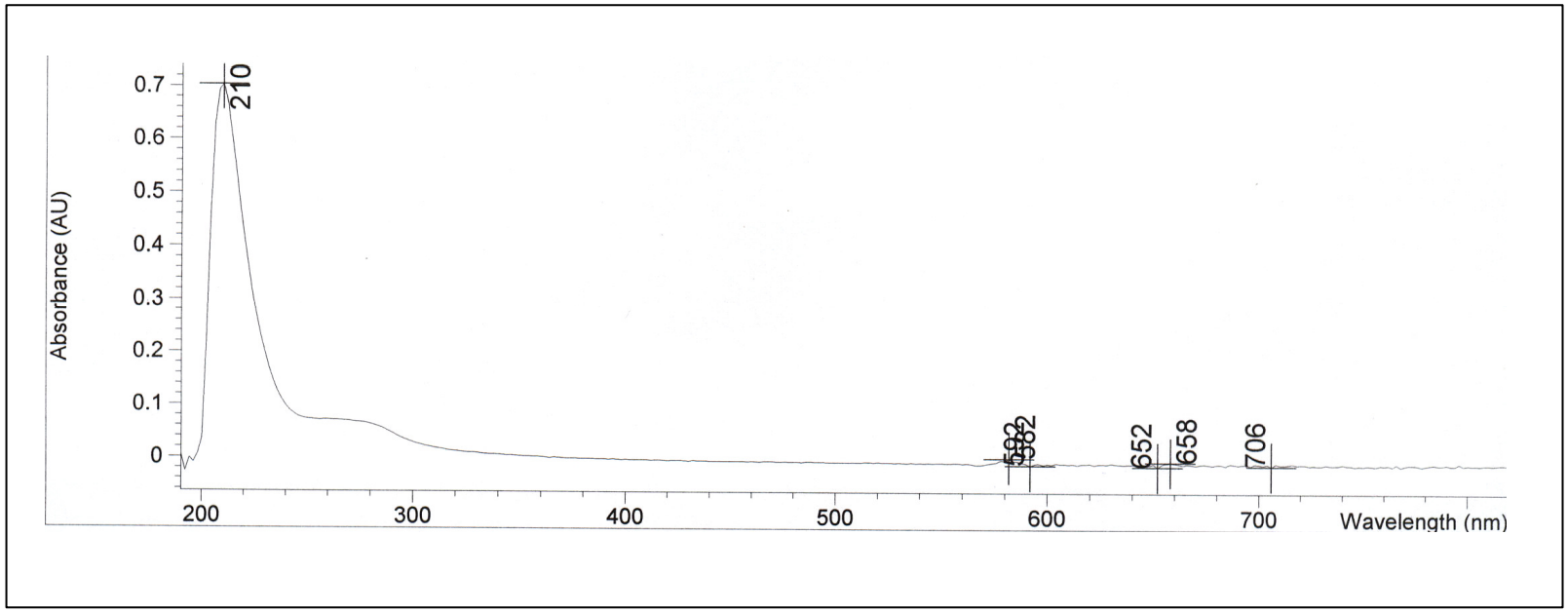
COSY spectrum of compound **G1**



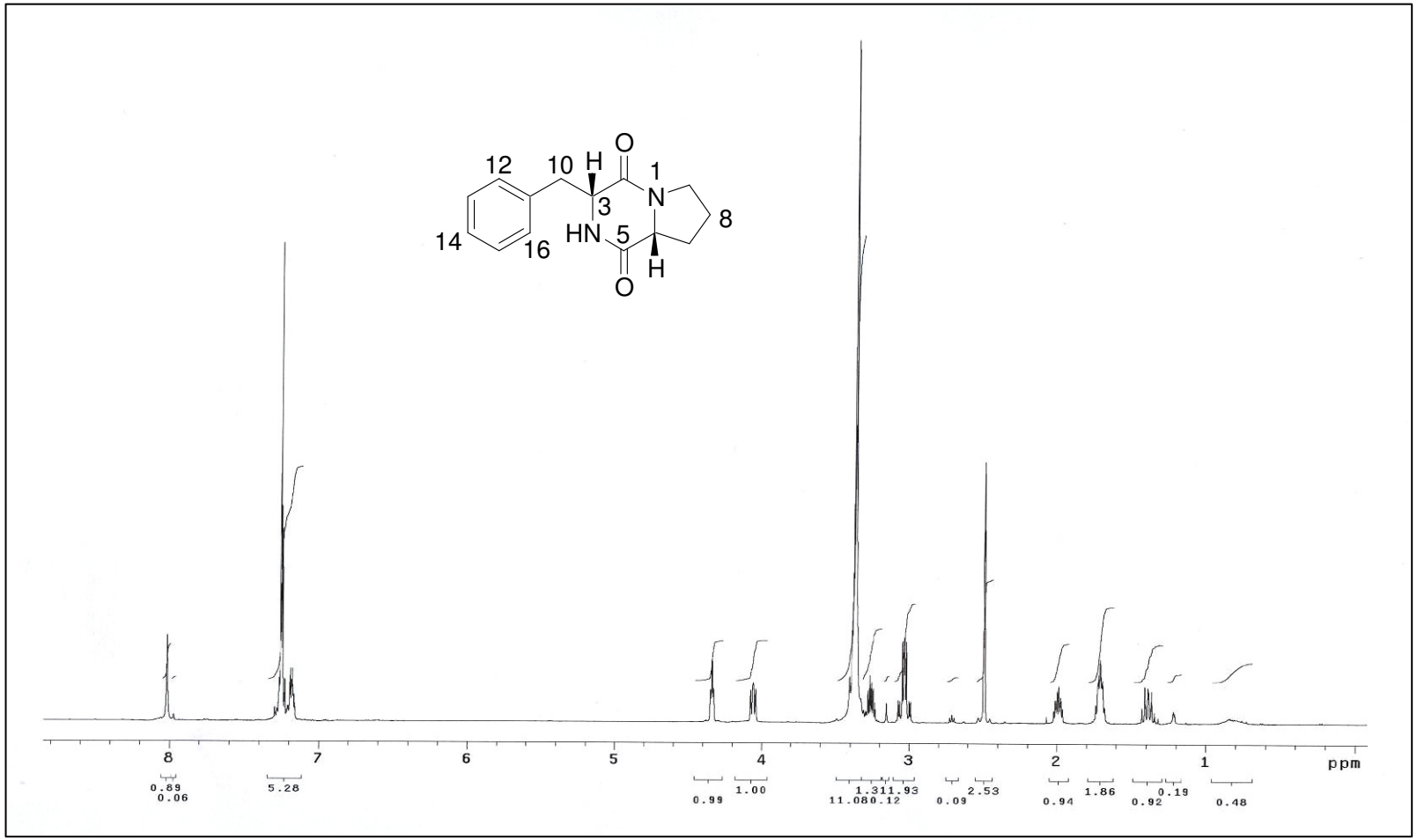
EIMS spectrum of compound **G1**



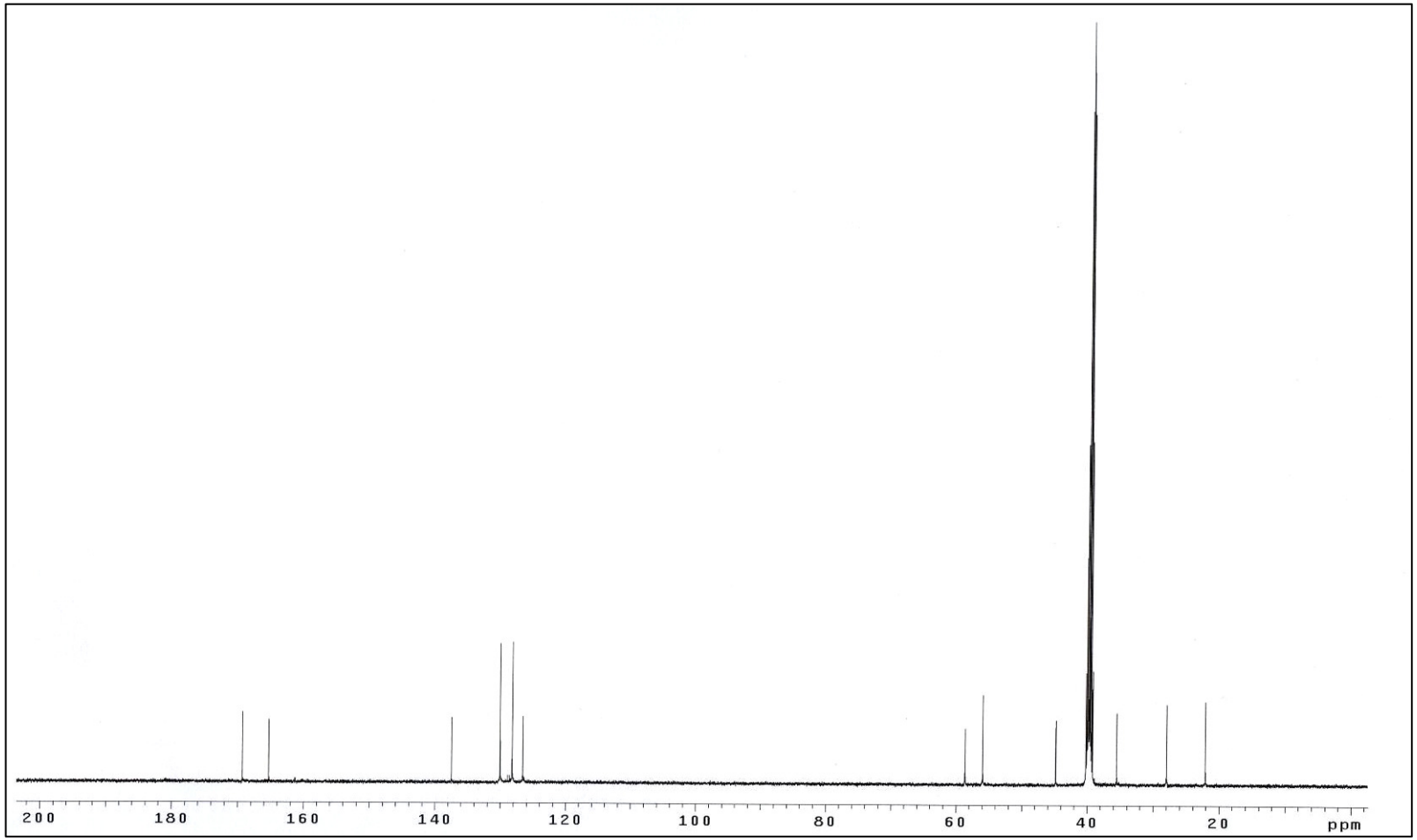
IR spectrum of compound **G1**



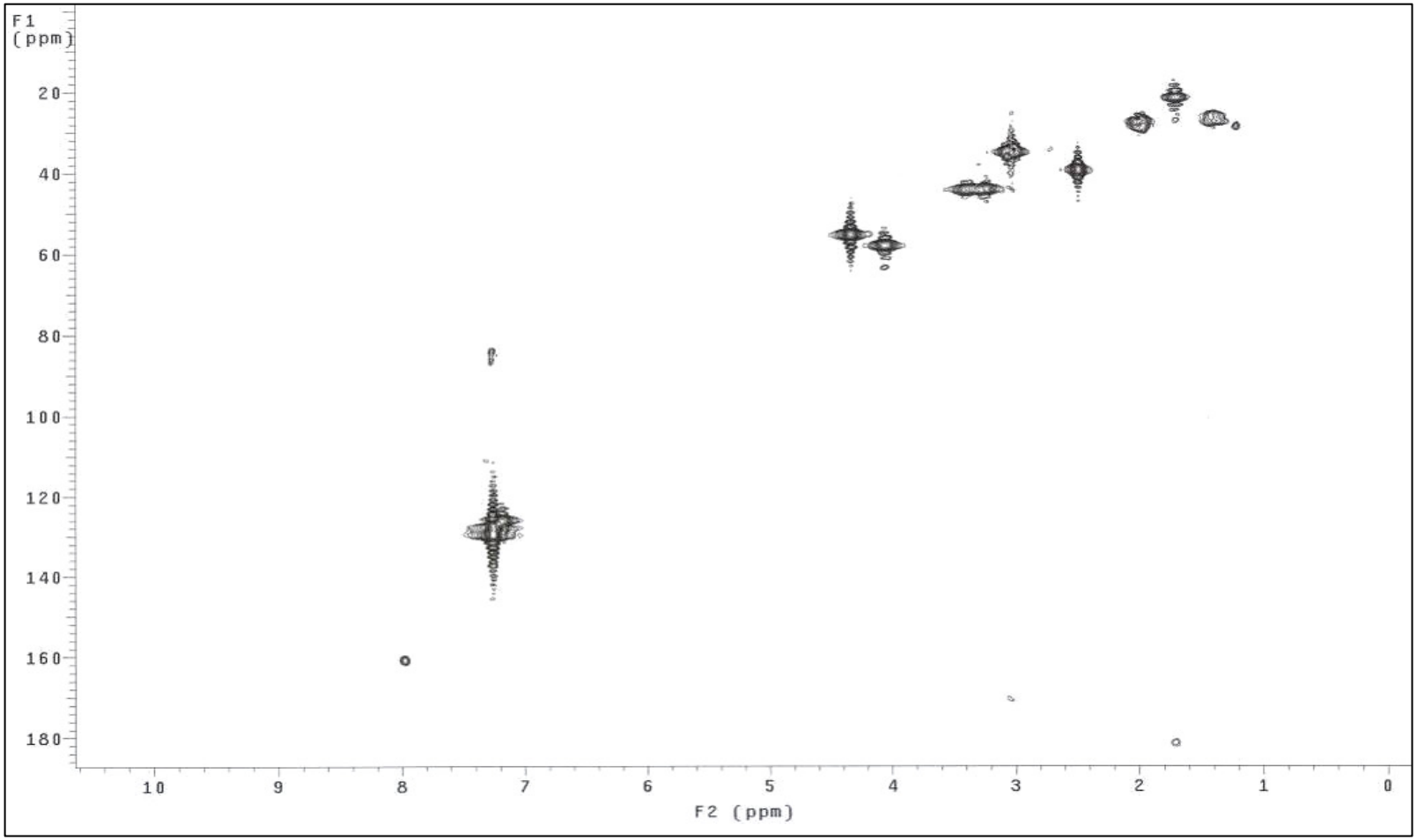
UV spectrum of compound **G1**



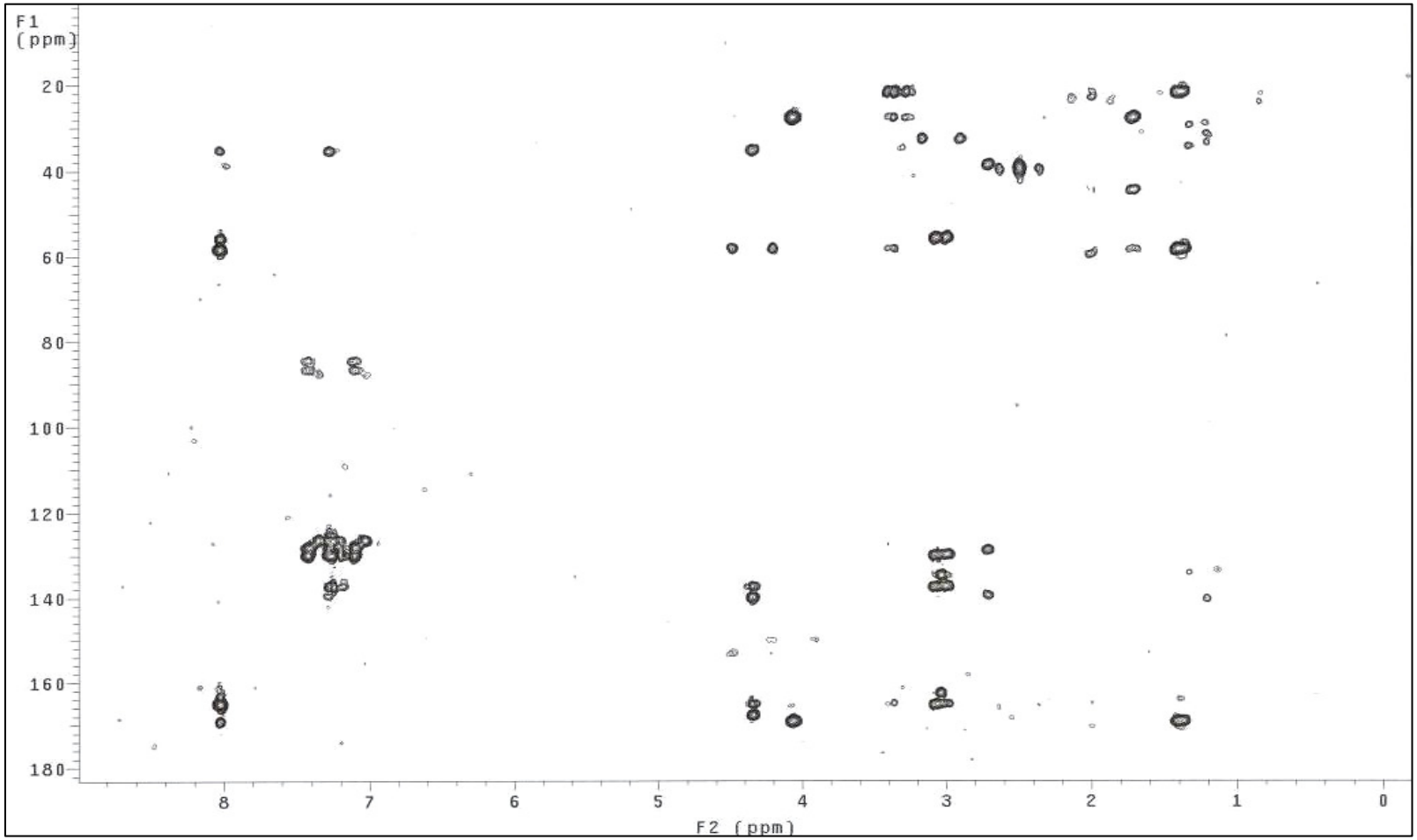
^{1}H NMR spectrum of compound **G2** (500 MHz, DMSO-d_6)



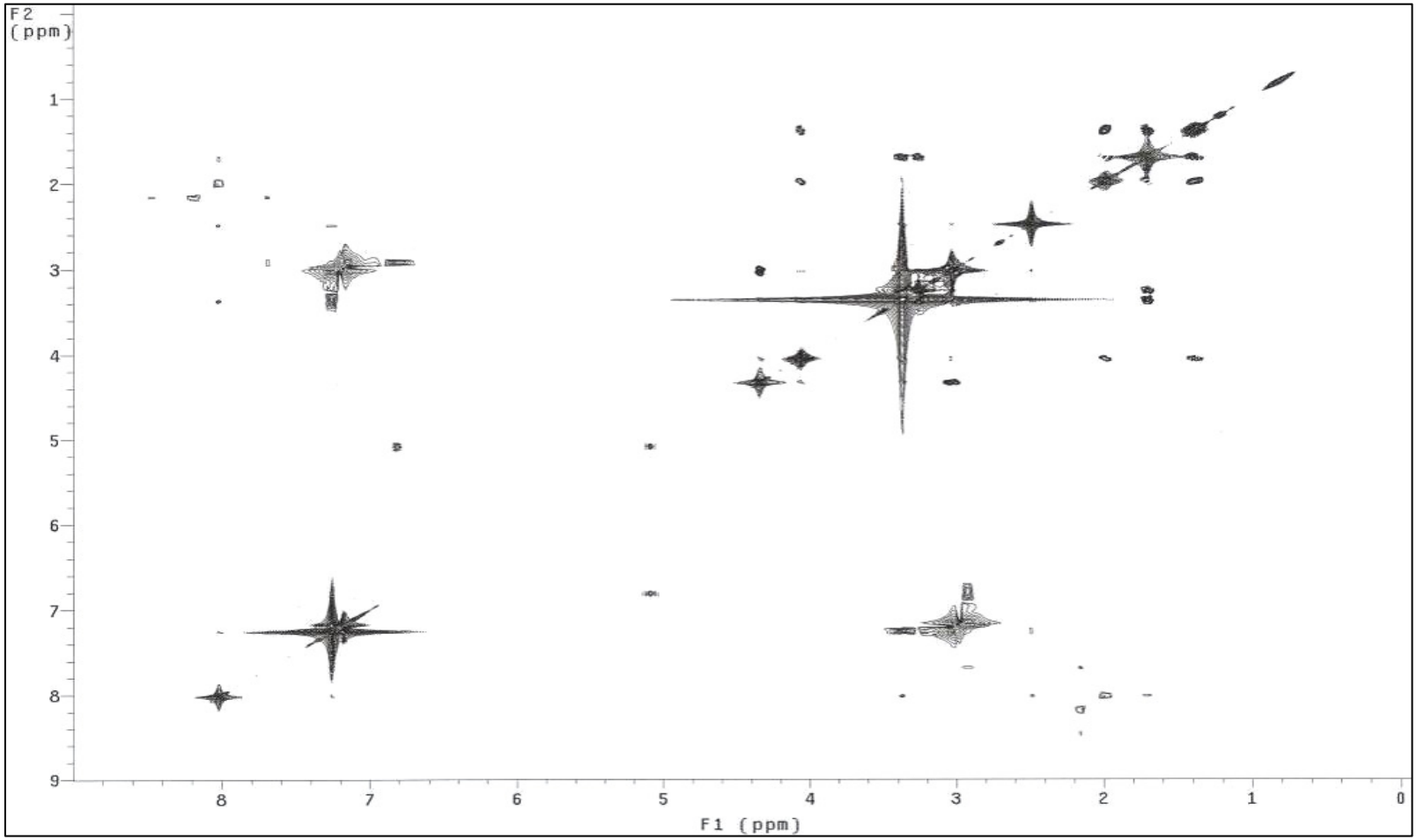
^{13}C NMR spectrum of compound **G2** (300 MHz, DMSO-d_6)



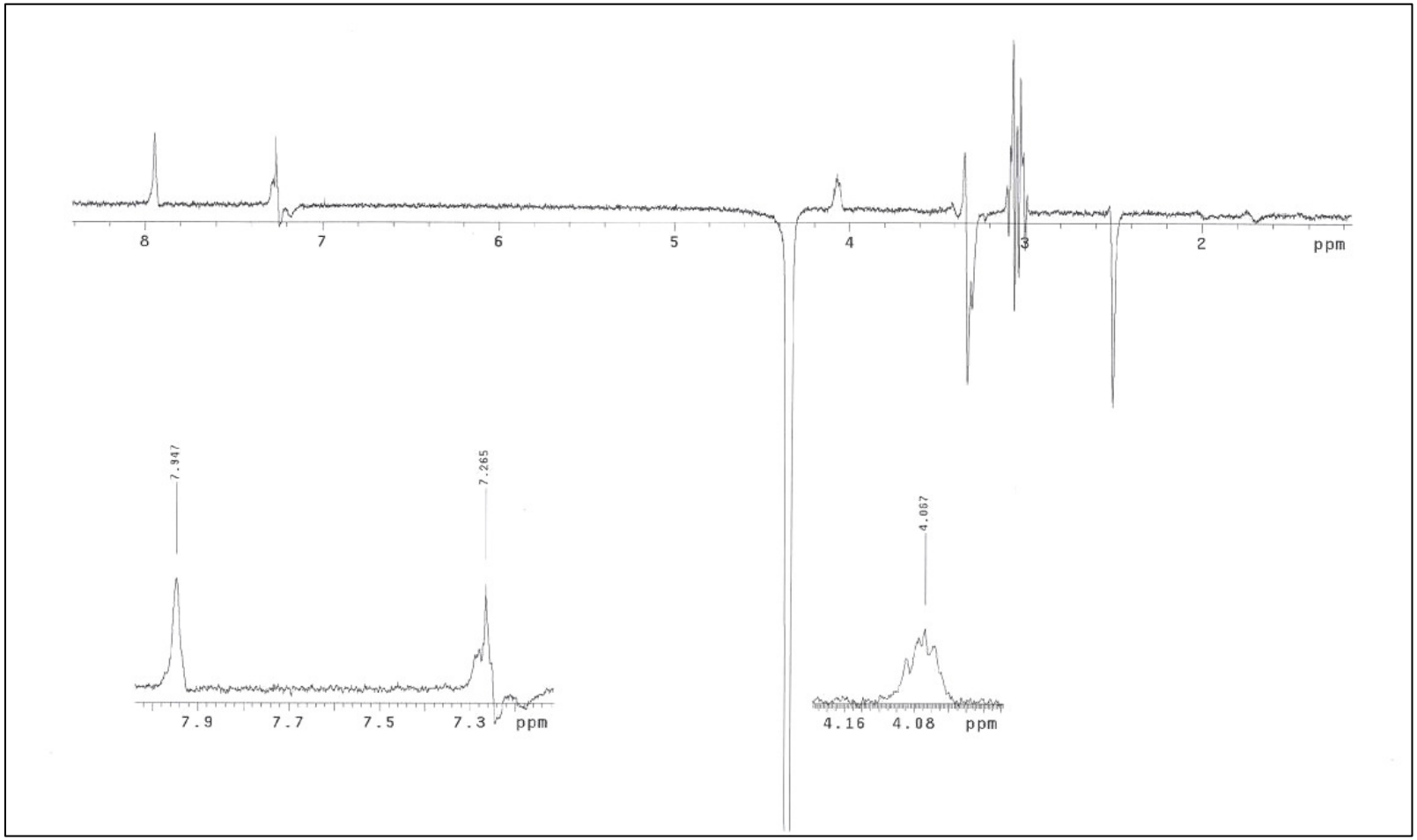
HMQC spectrum of compound **G2**



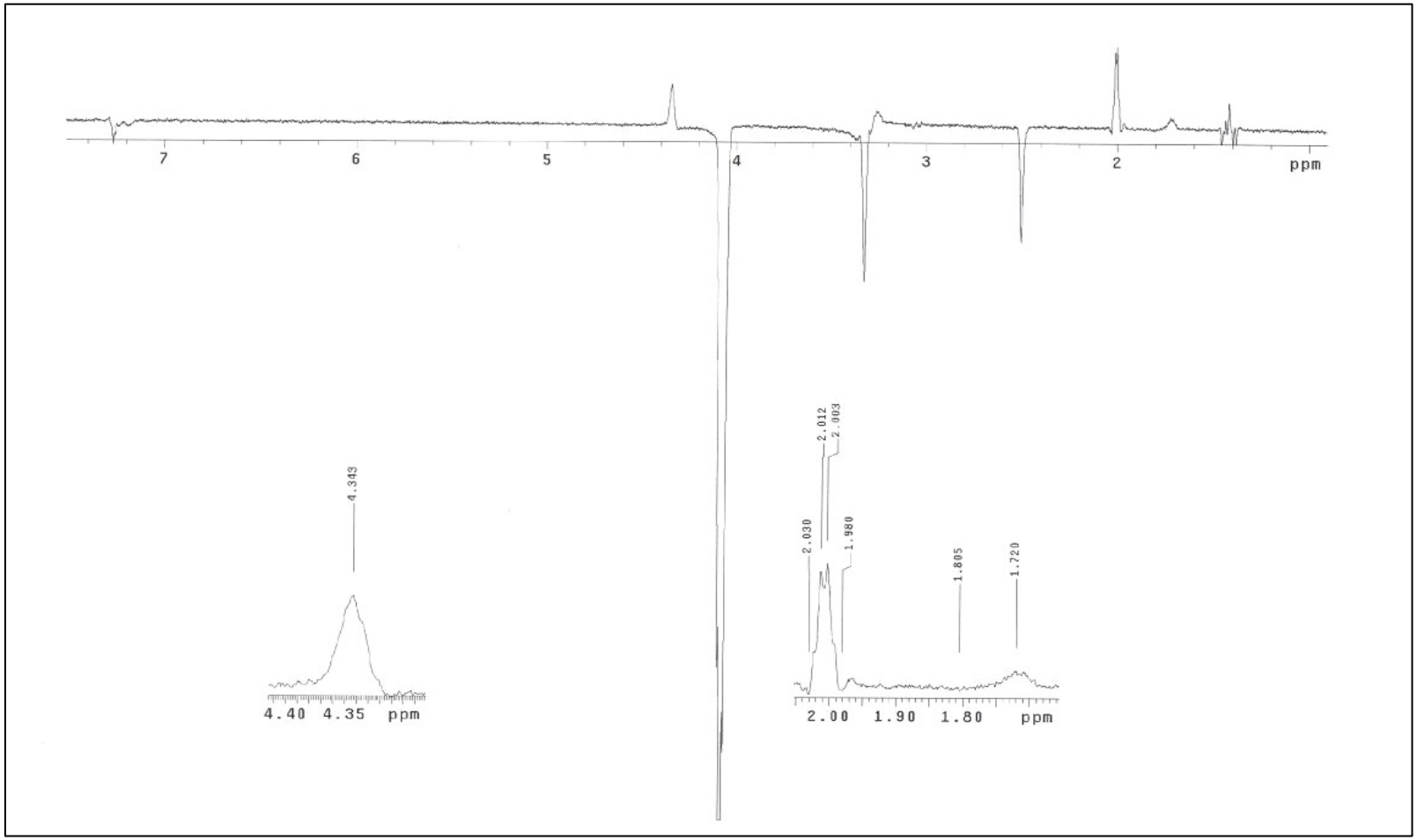
HMBC spectrum of compound **G2**



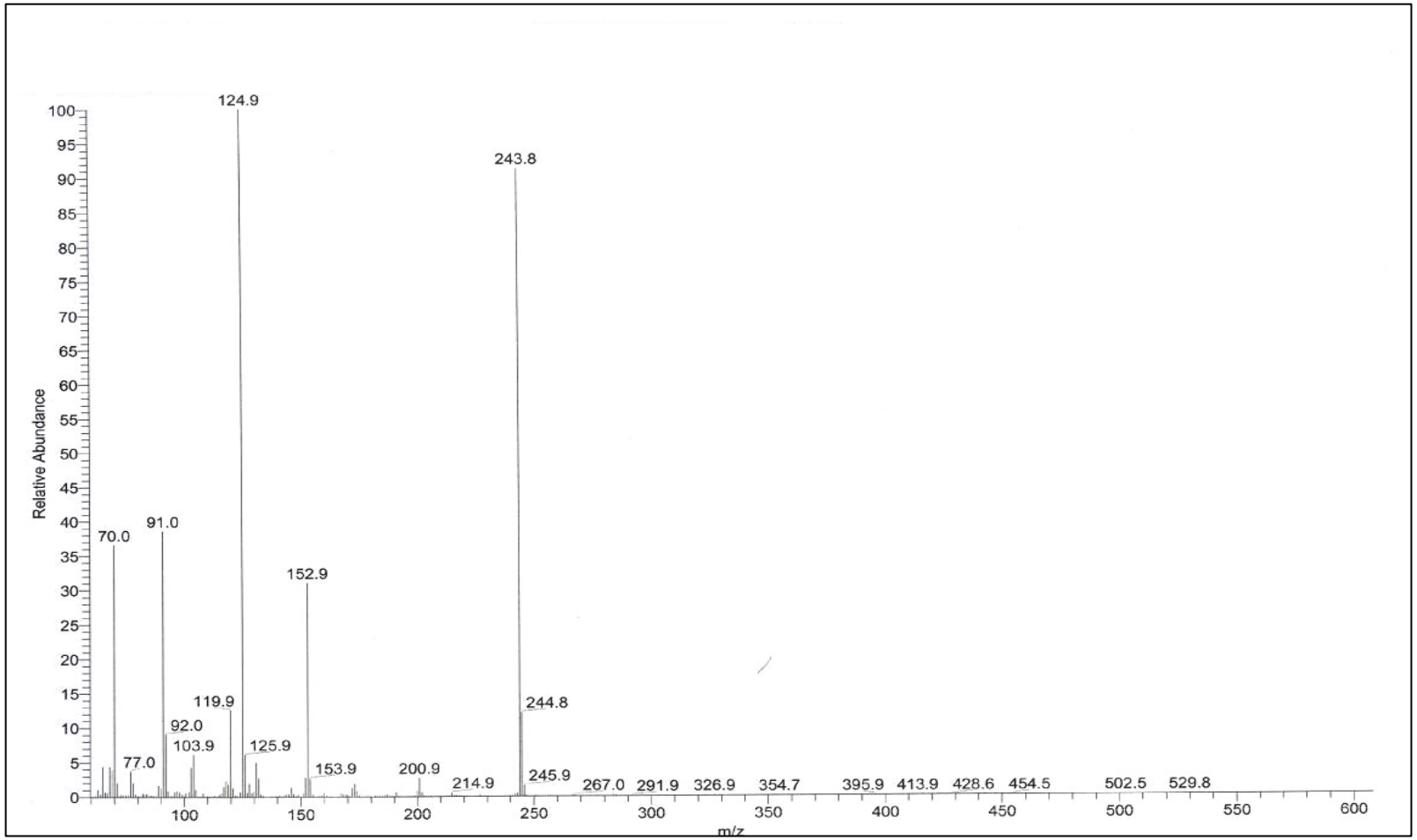
COSY spectrum of compound **G2**



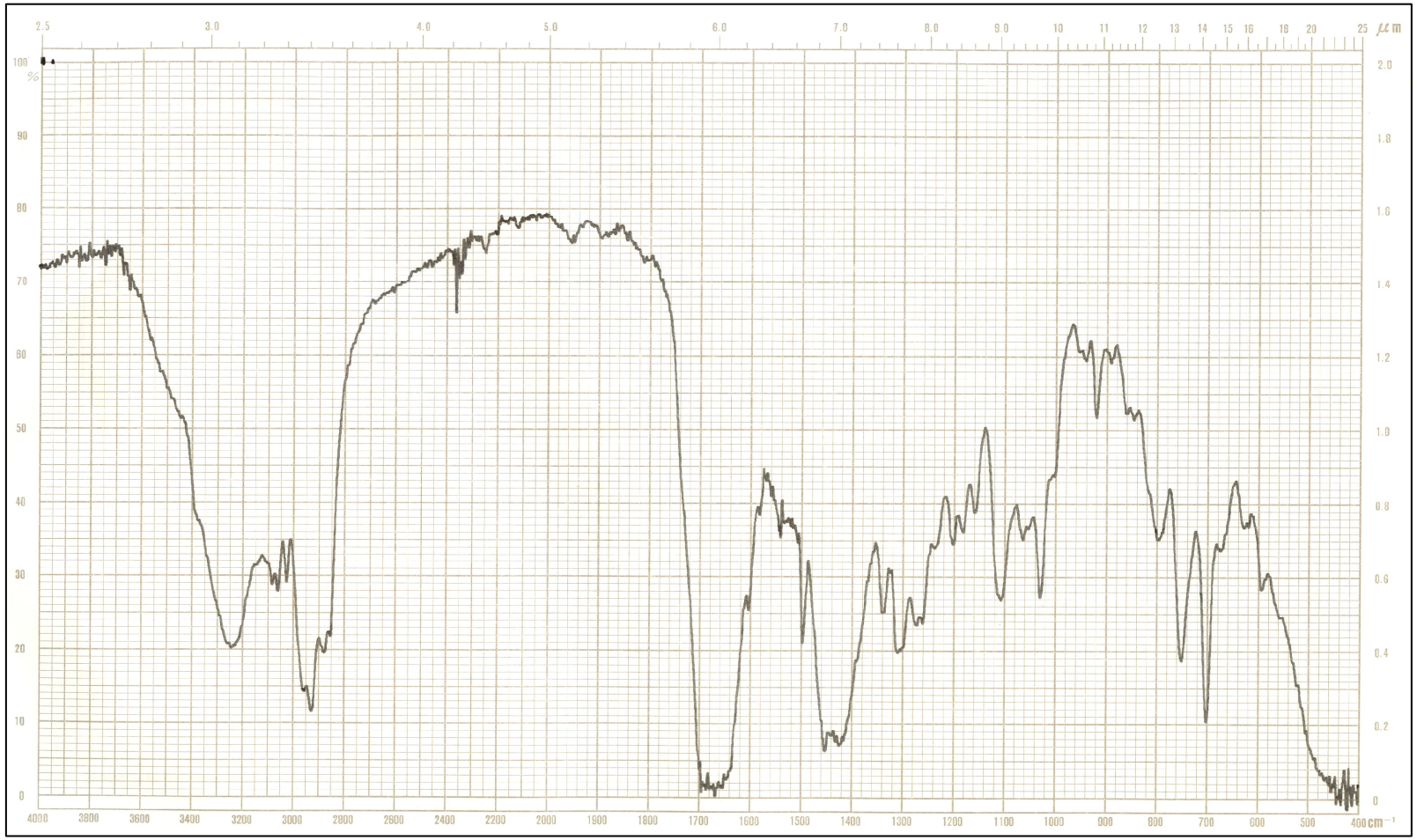
NOE spectrum of compound **G2**



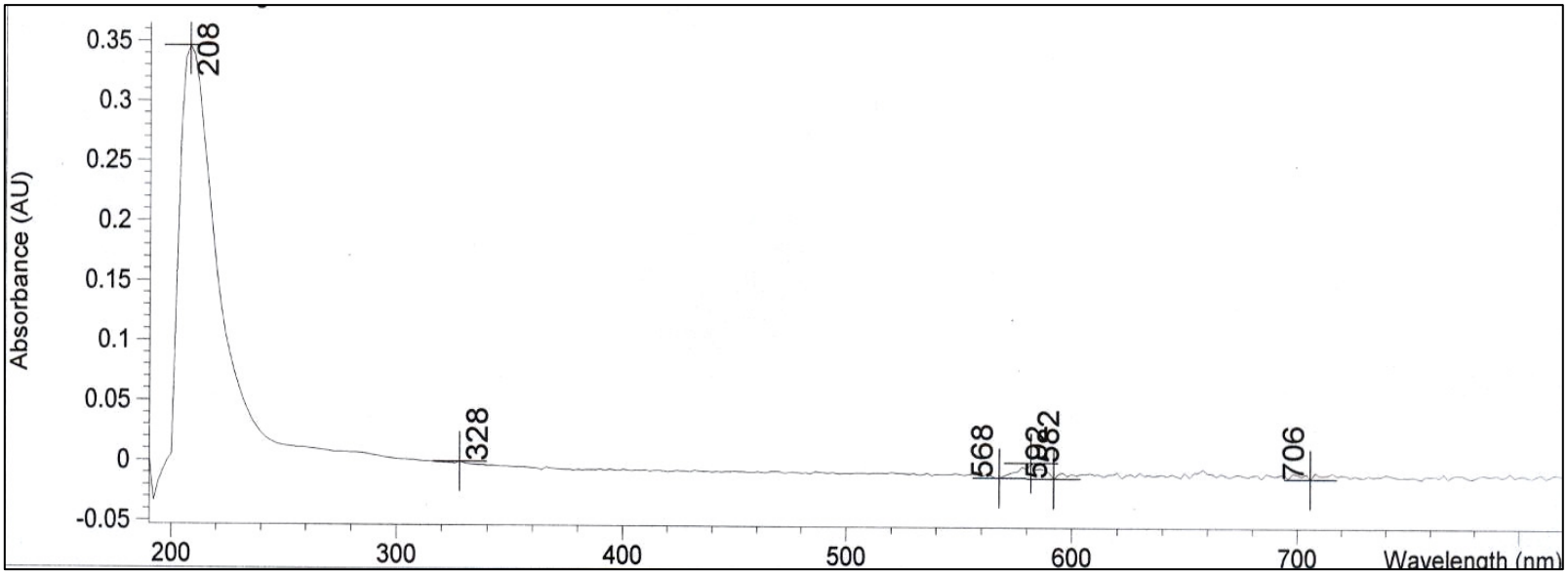
NOE spectrum of compound **G2**



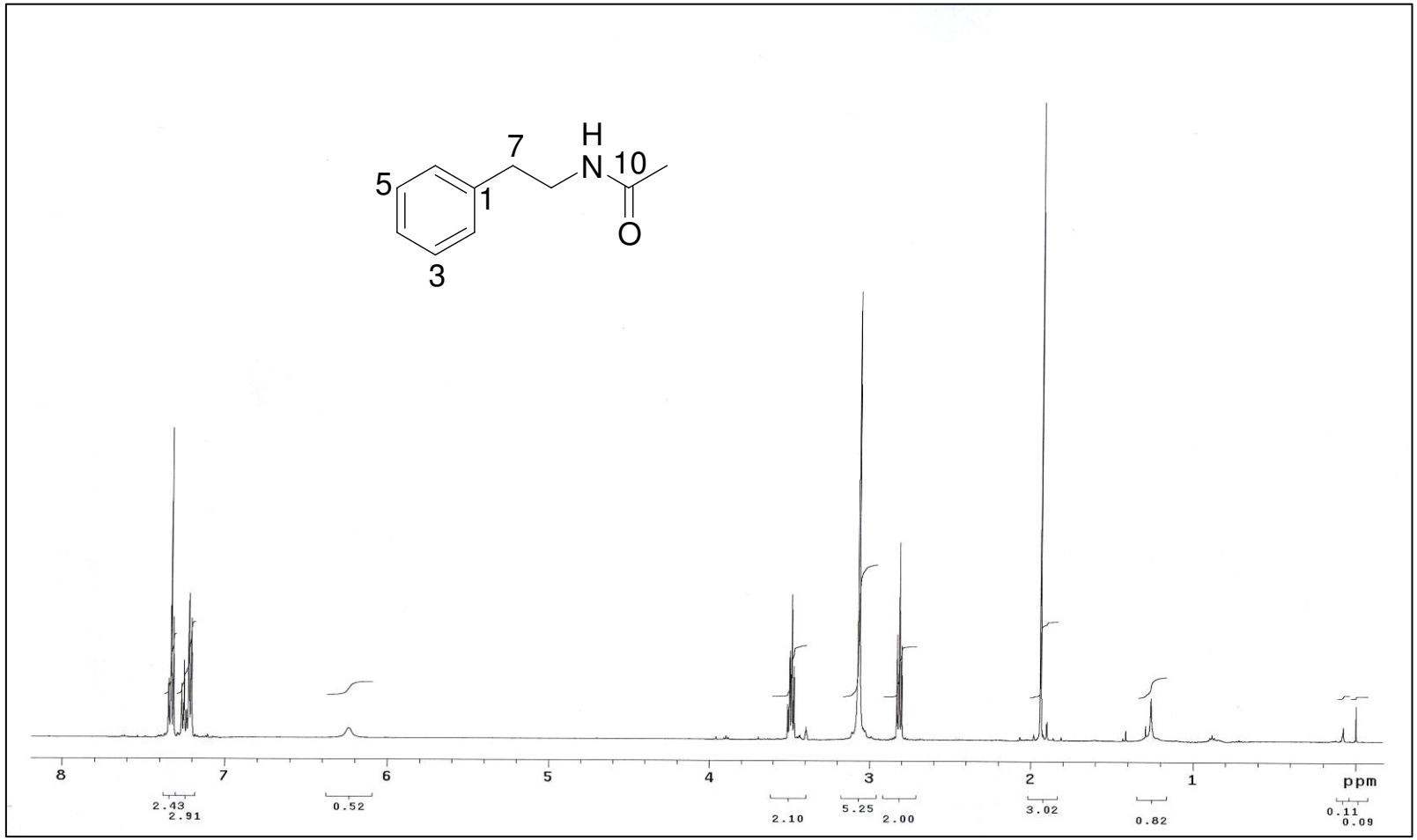
EIMS spectrum of compound **G2**



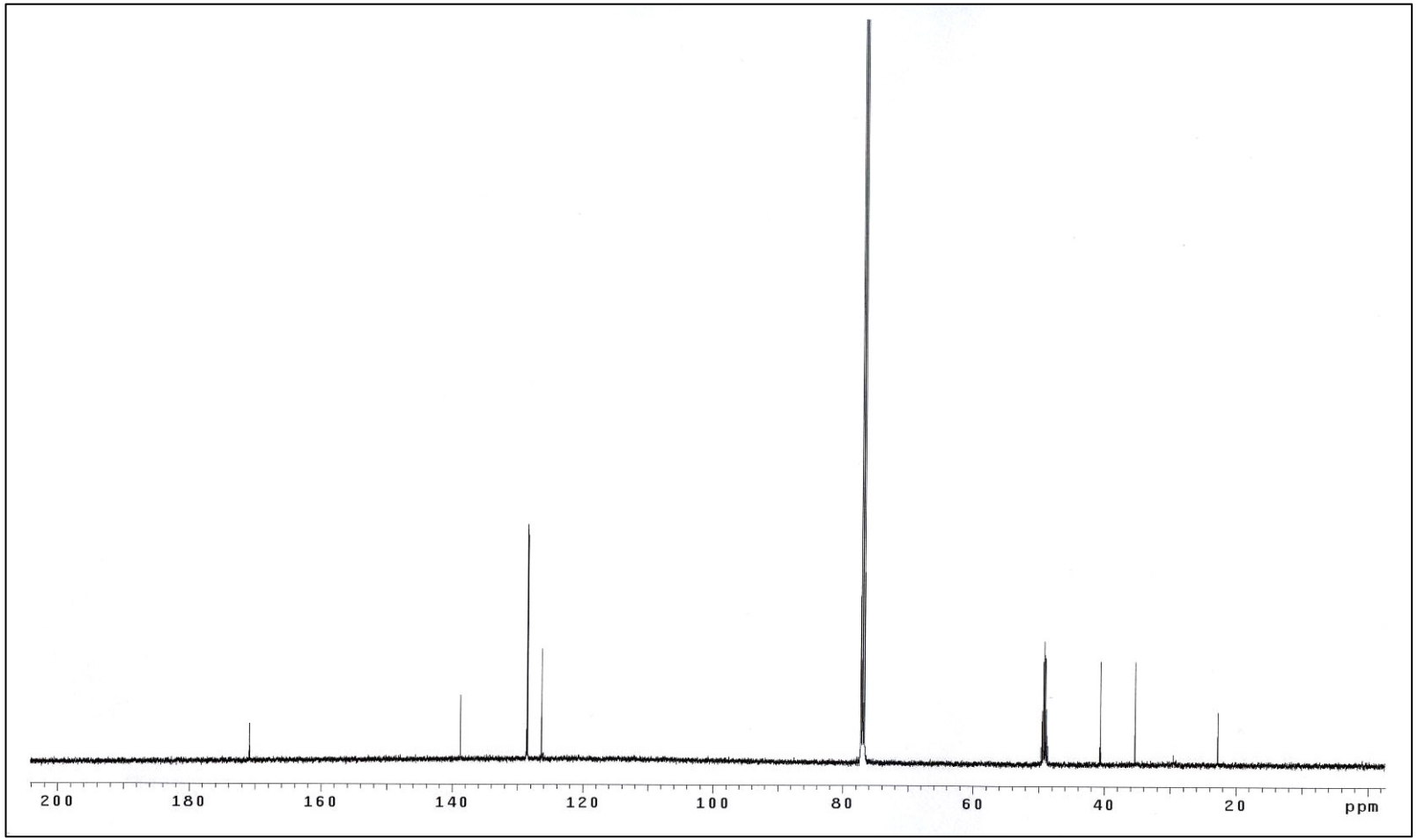
IR spectrum of compound **G2**



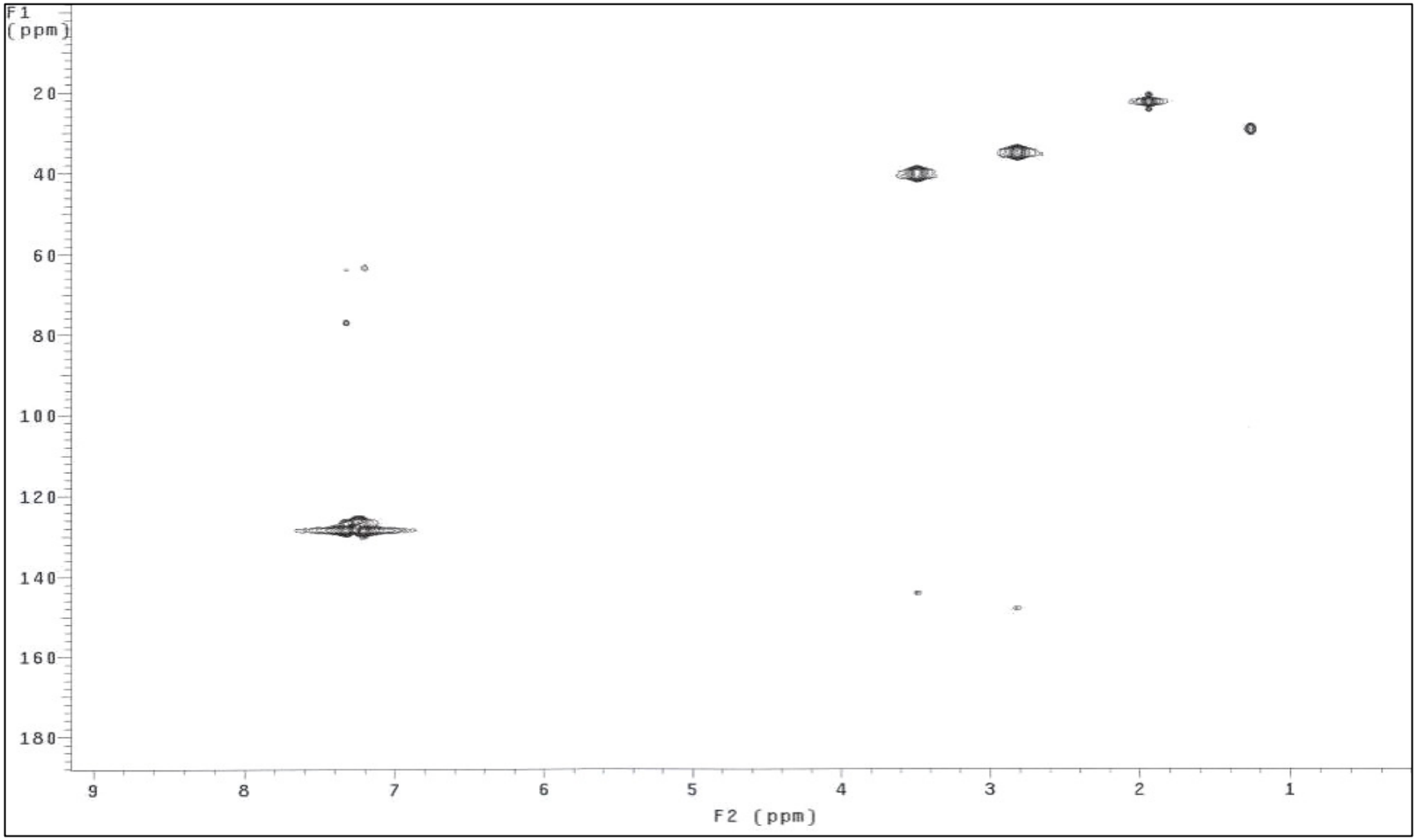
UV spectrum of compound **G2**



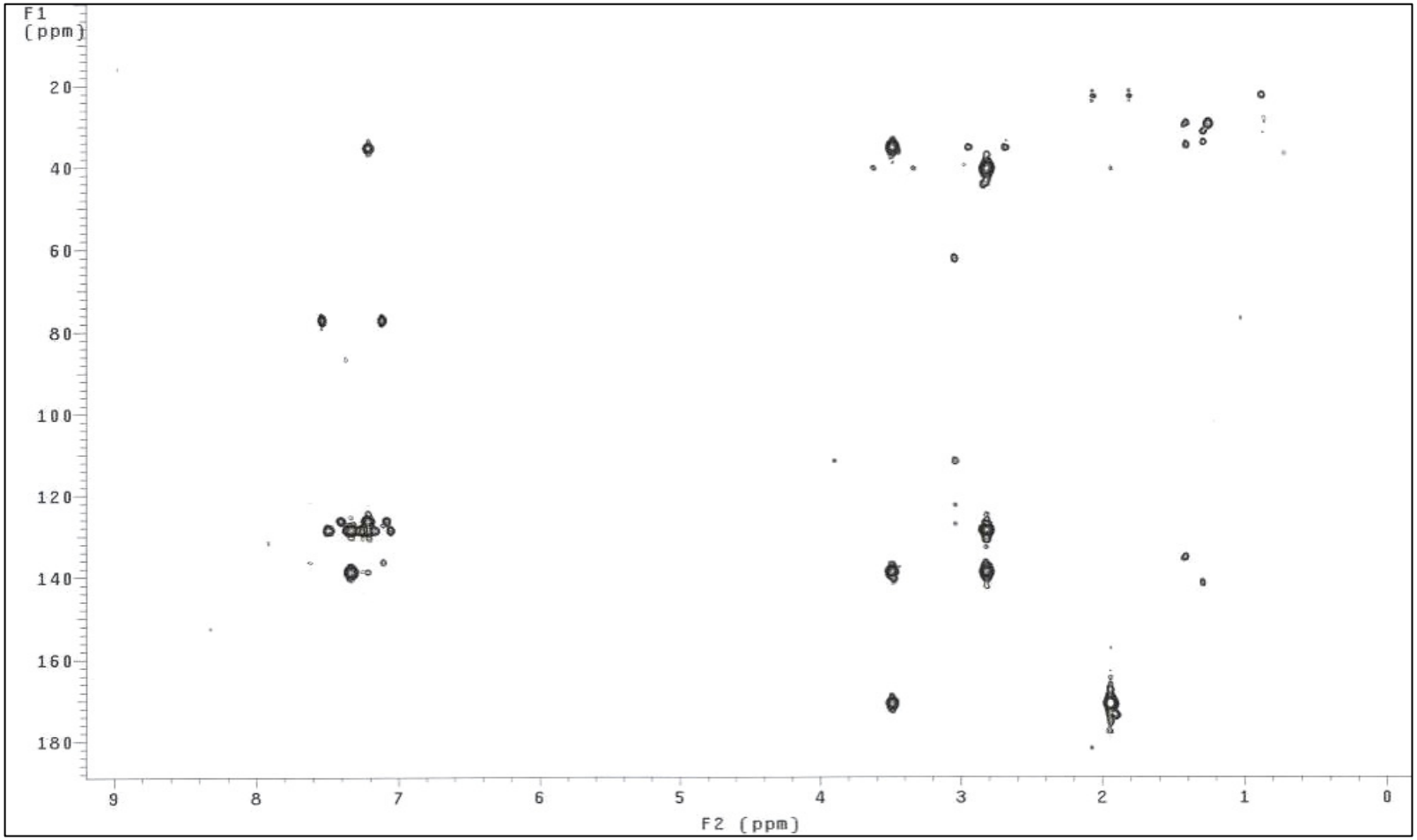
^{1}H NMR spectrum of compound **G3** (500 MHz, $CDCl_3$+CD_3OD)



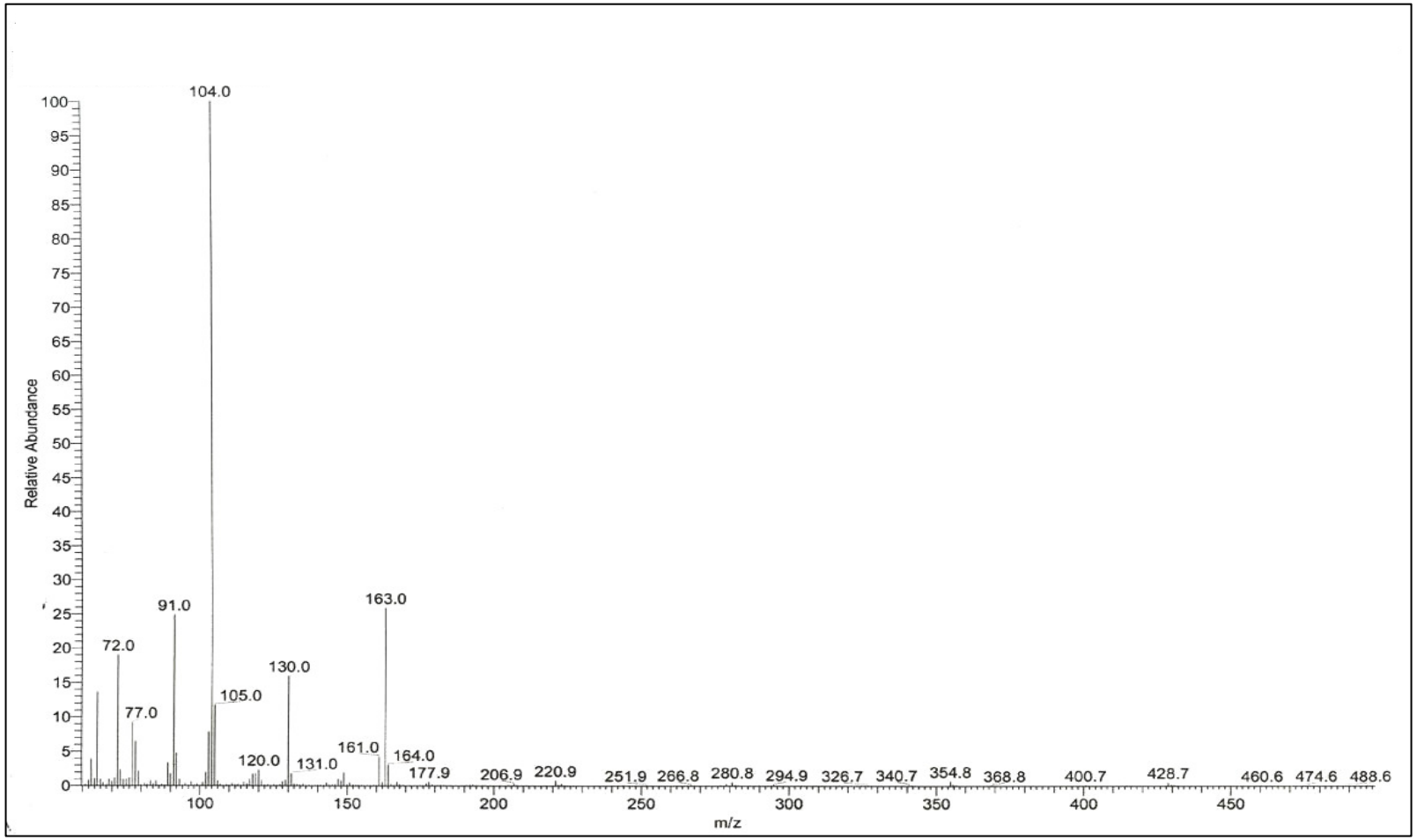
^{13}C NMR spectrum of compound **G3** (300 MHz, $CDCl_3$+CD_3OD)



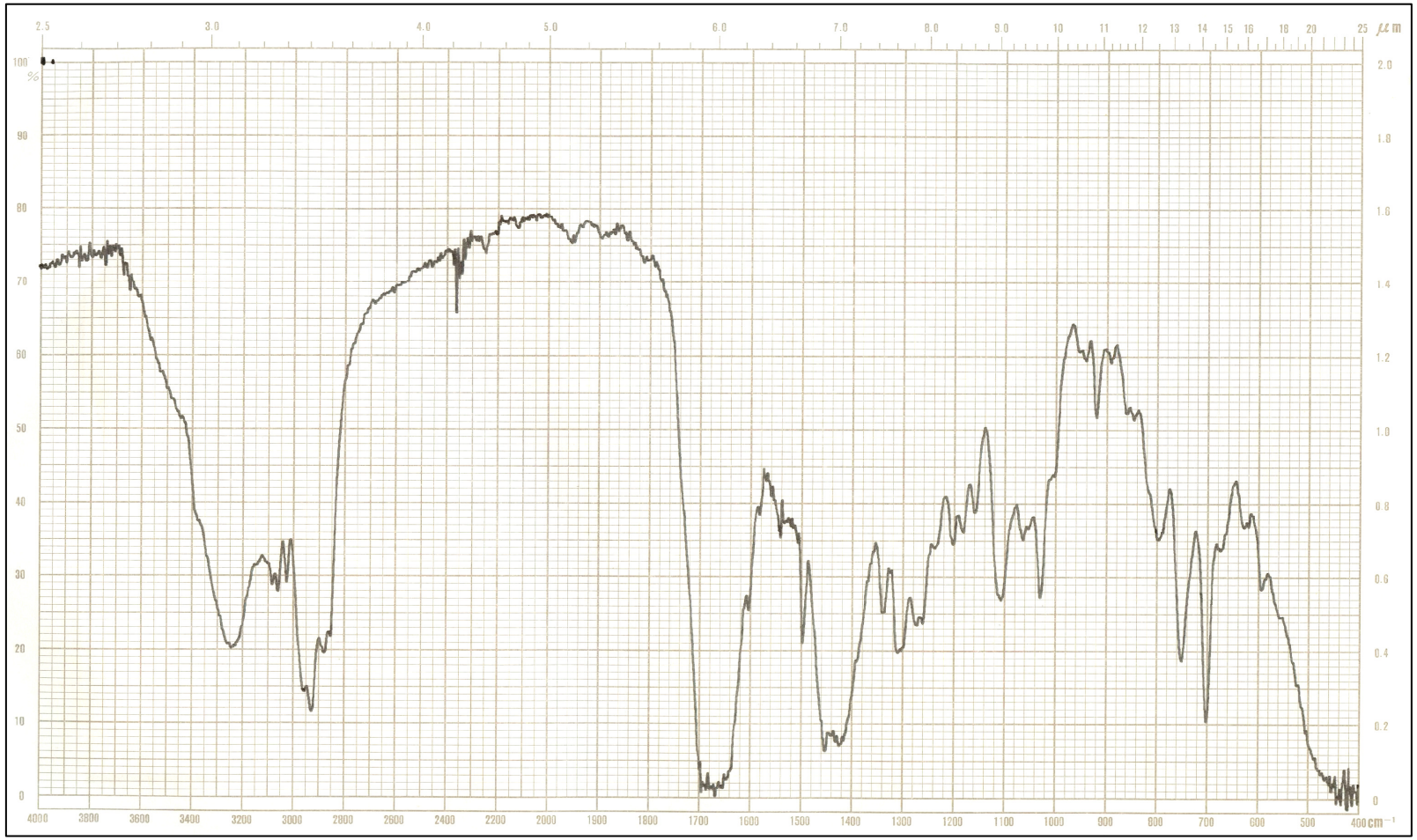
HMQC spectrum of compound **G3**



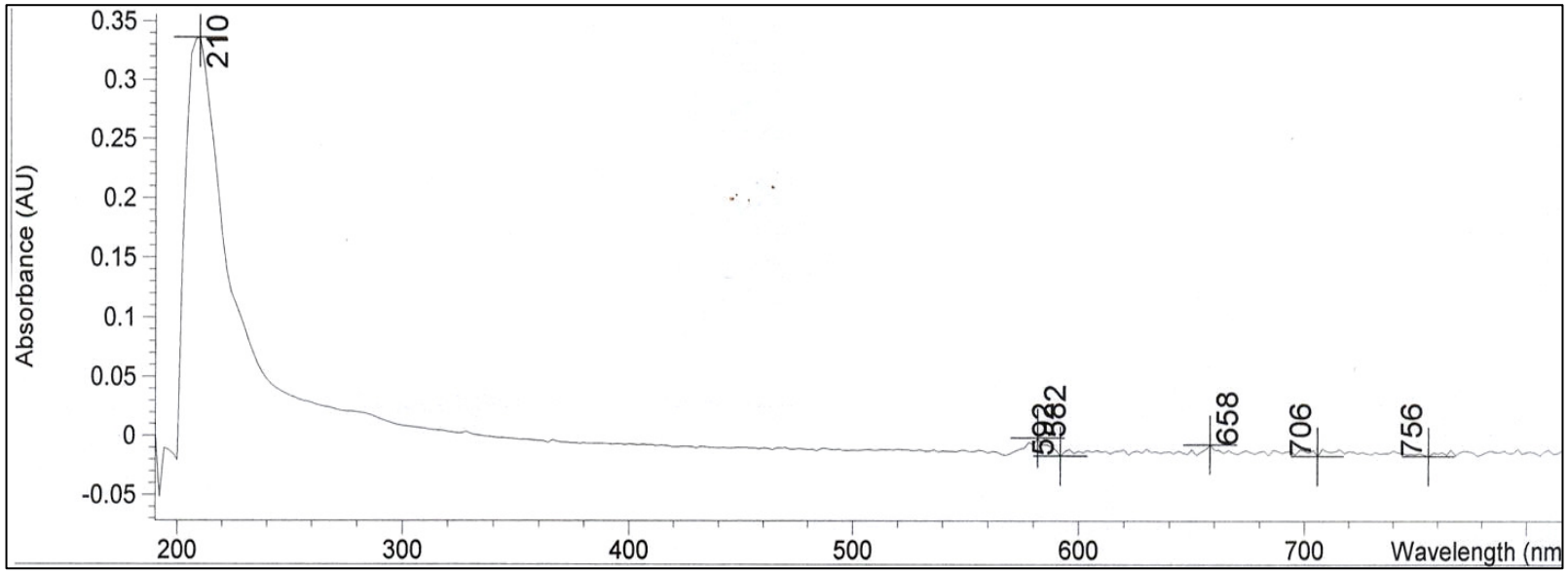
HMBC spectrum of compound **G3**



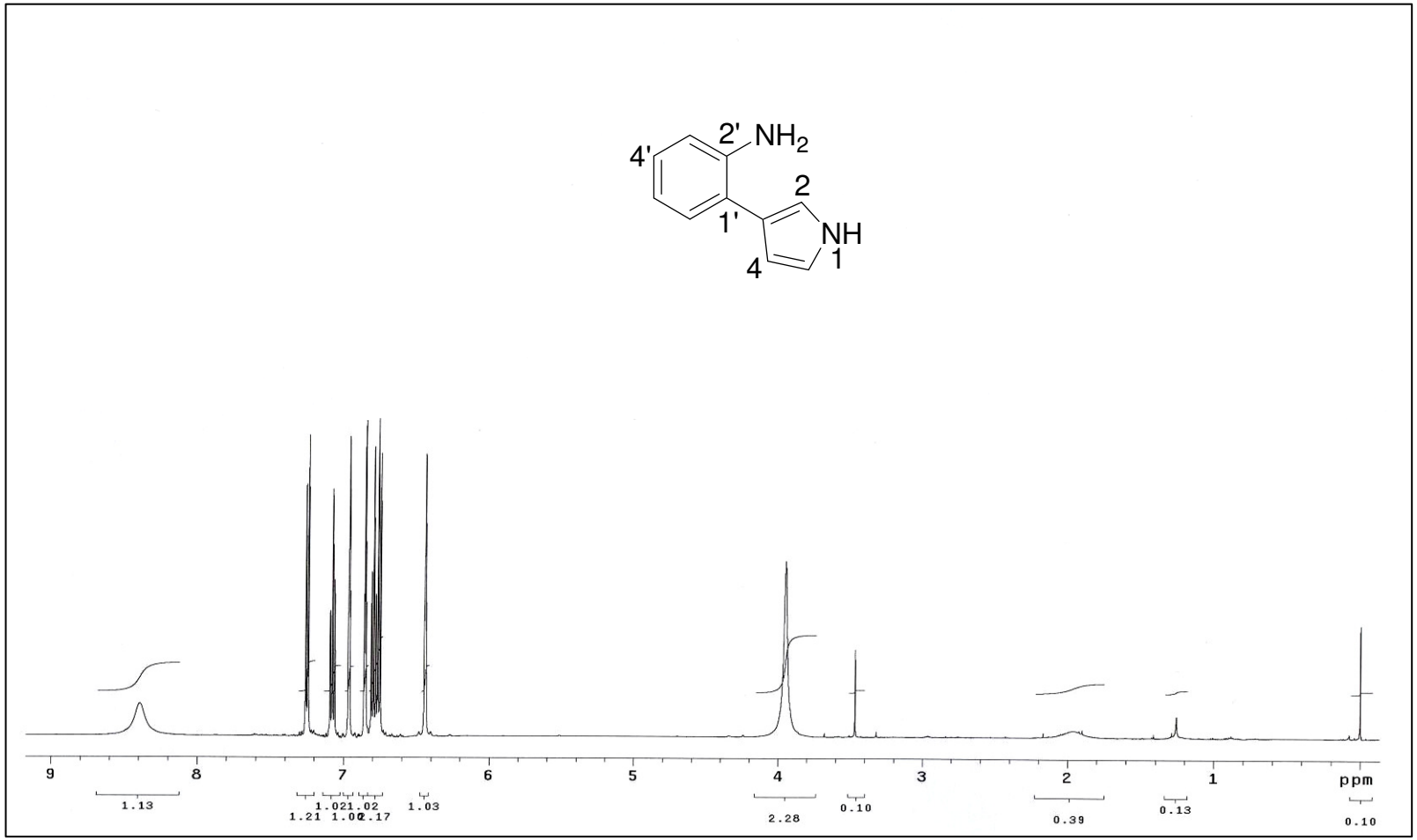
EIMS spectrum of compound **G3**



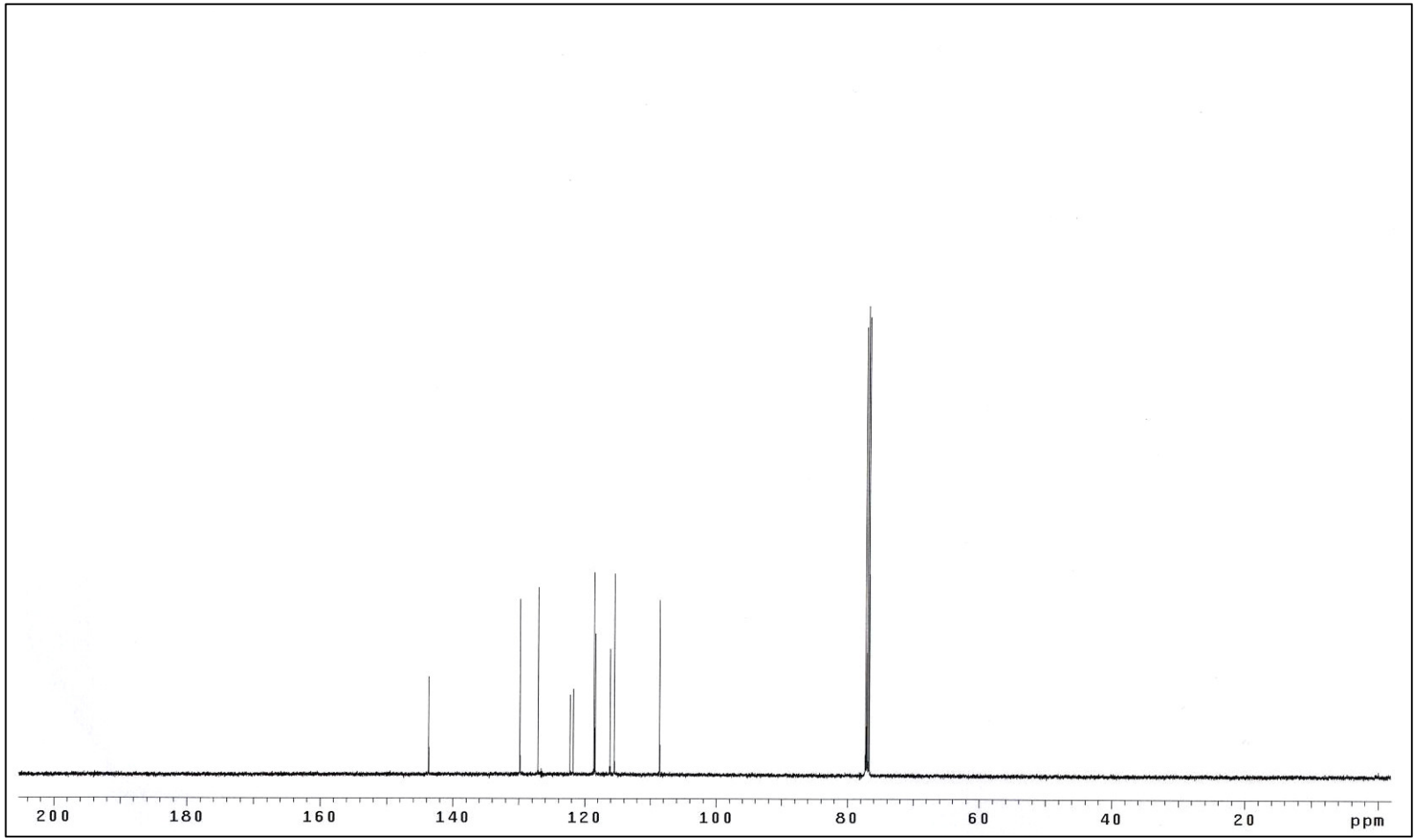
IR spectrum of compound **G3**



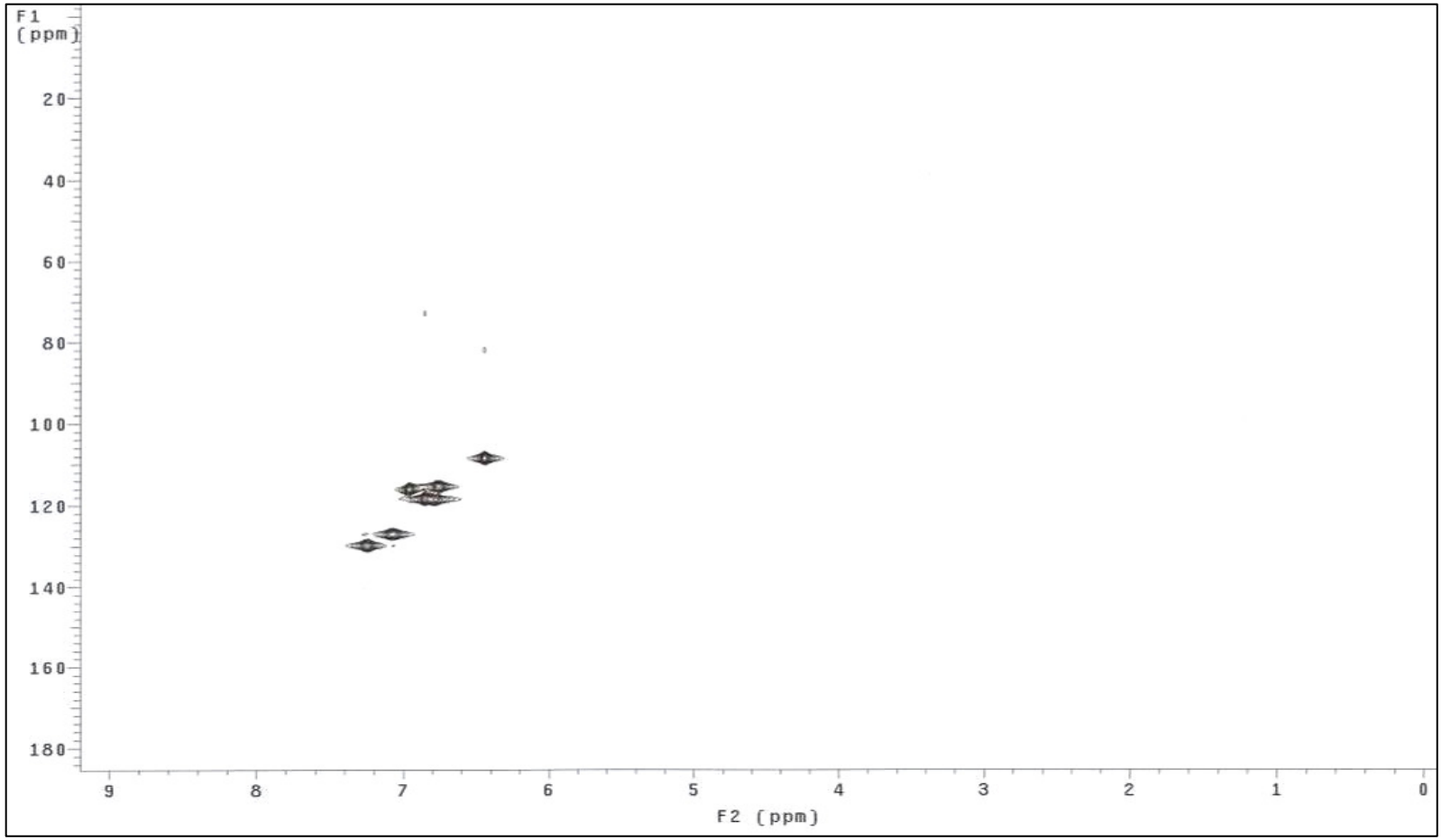
UV spectrum of compound **G3**



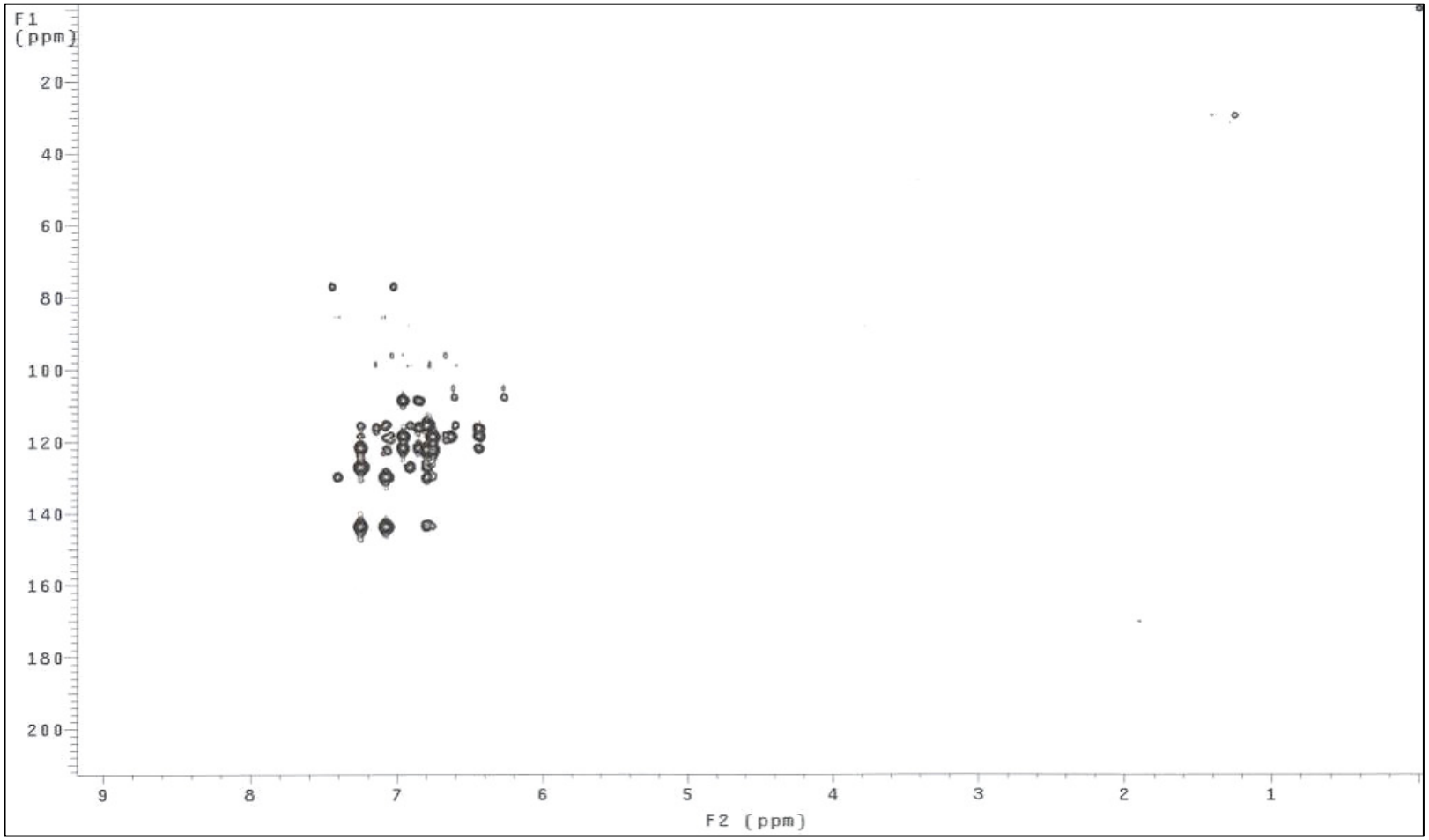
^{1}H NMR spectrum of compound **G4** (500 MHz, $CDCl_3$)



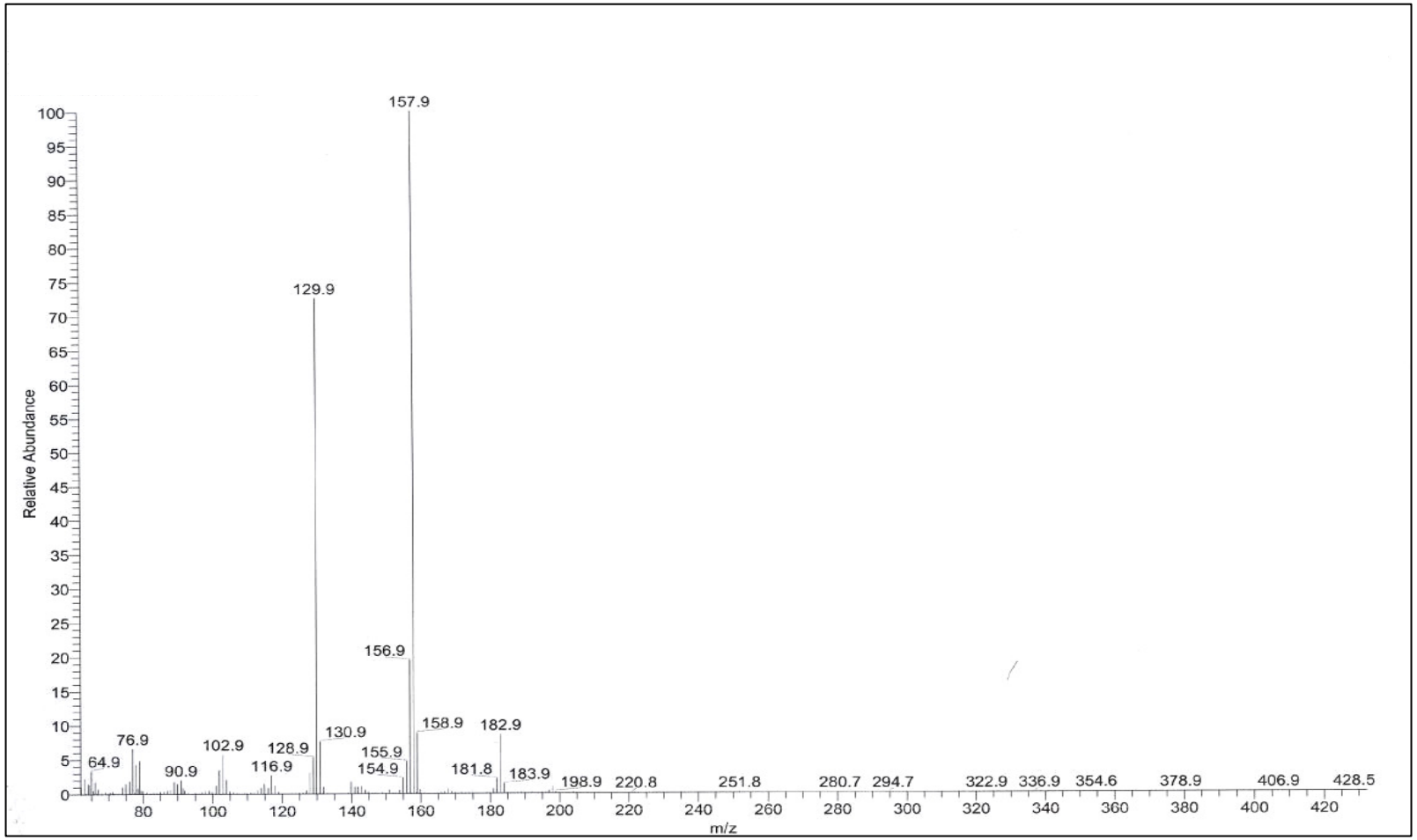
^{13}C NMR spectrum of compound **G4** (300 MHz, $CDCl_3$)



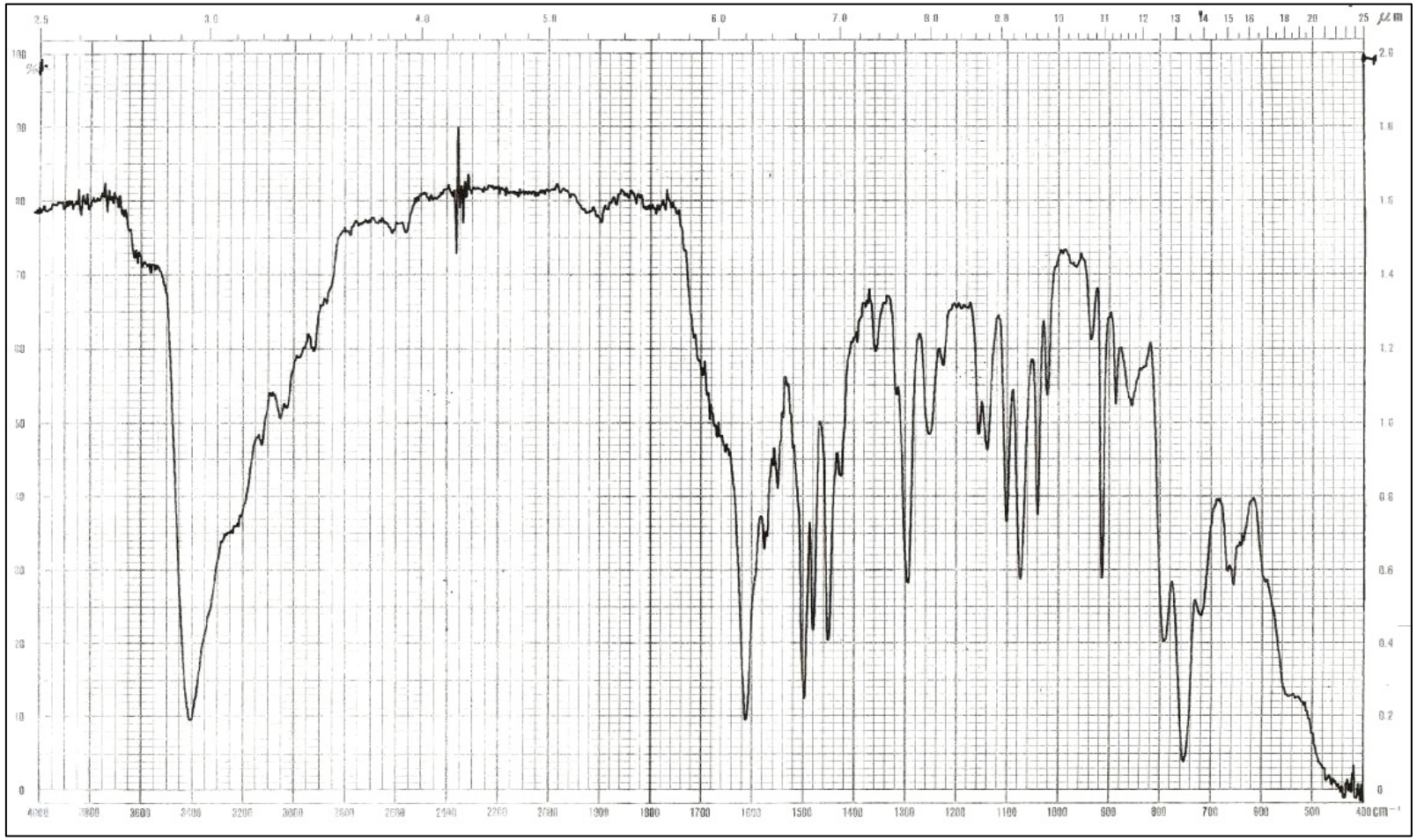
HMQC spectrum of compound **G4**



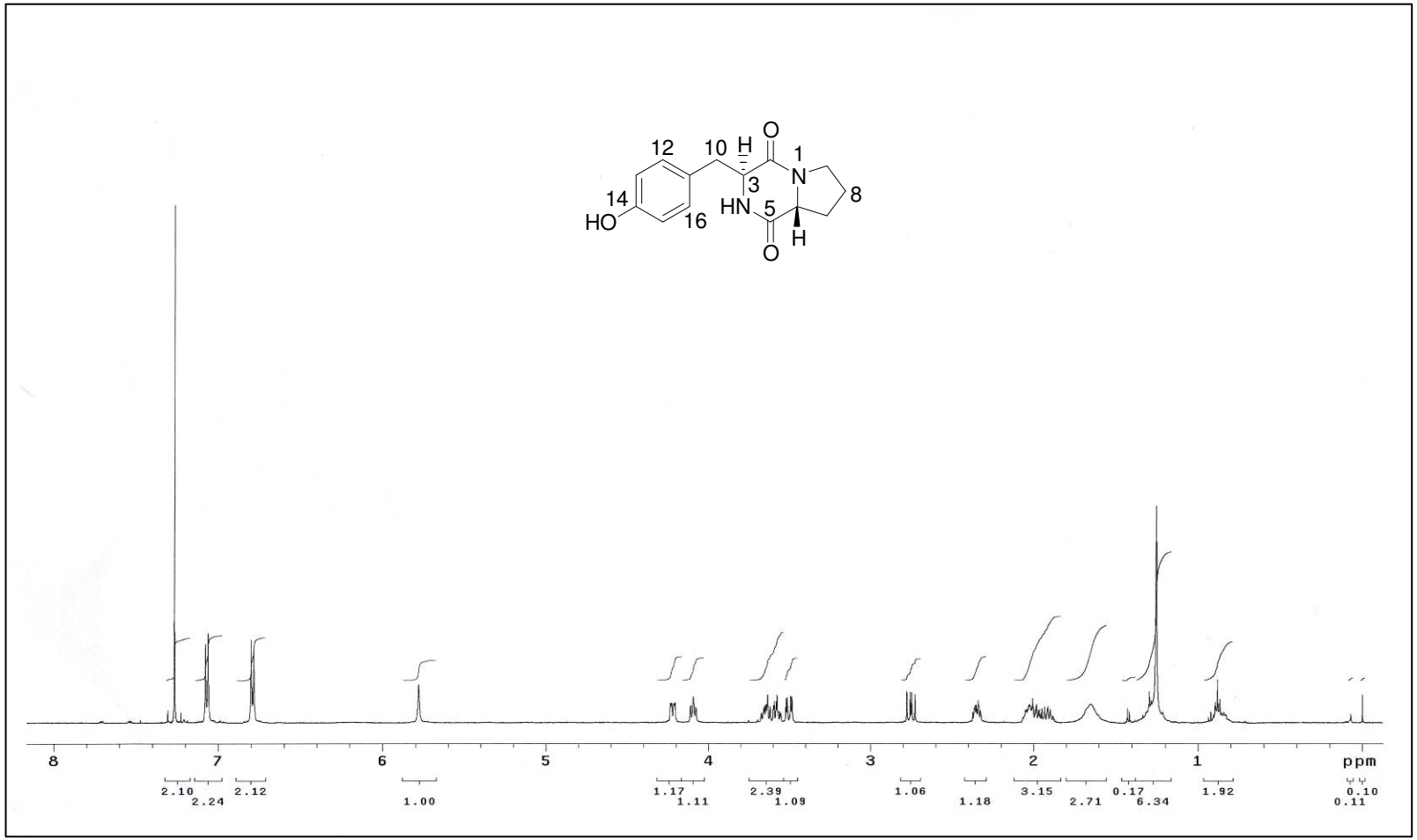
HMBC spectrum of compound **G4**



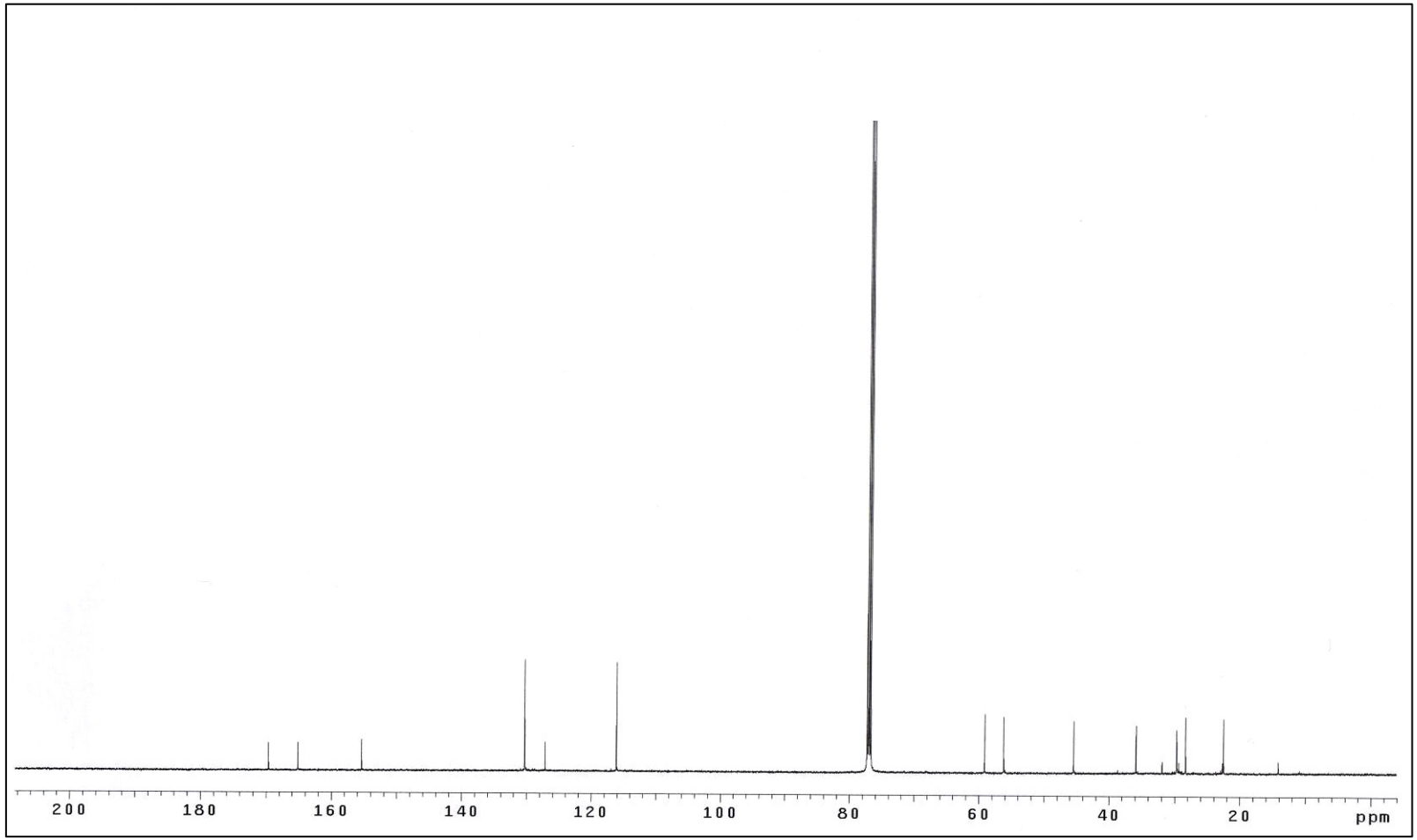
EIMS spectrum of compound **G4**



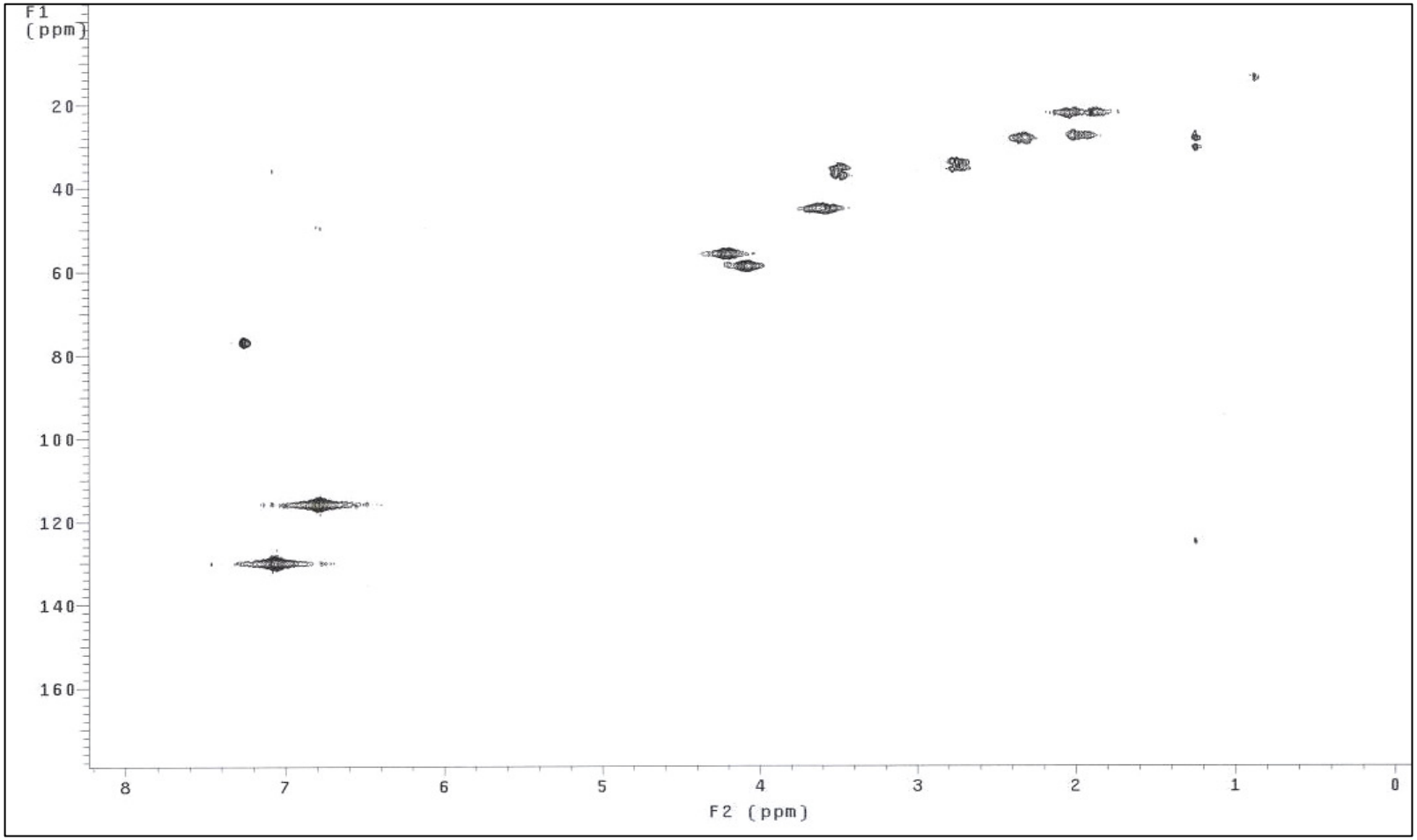
IR spectrum of compound **G4**



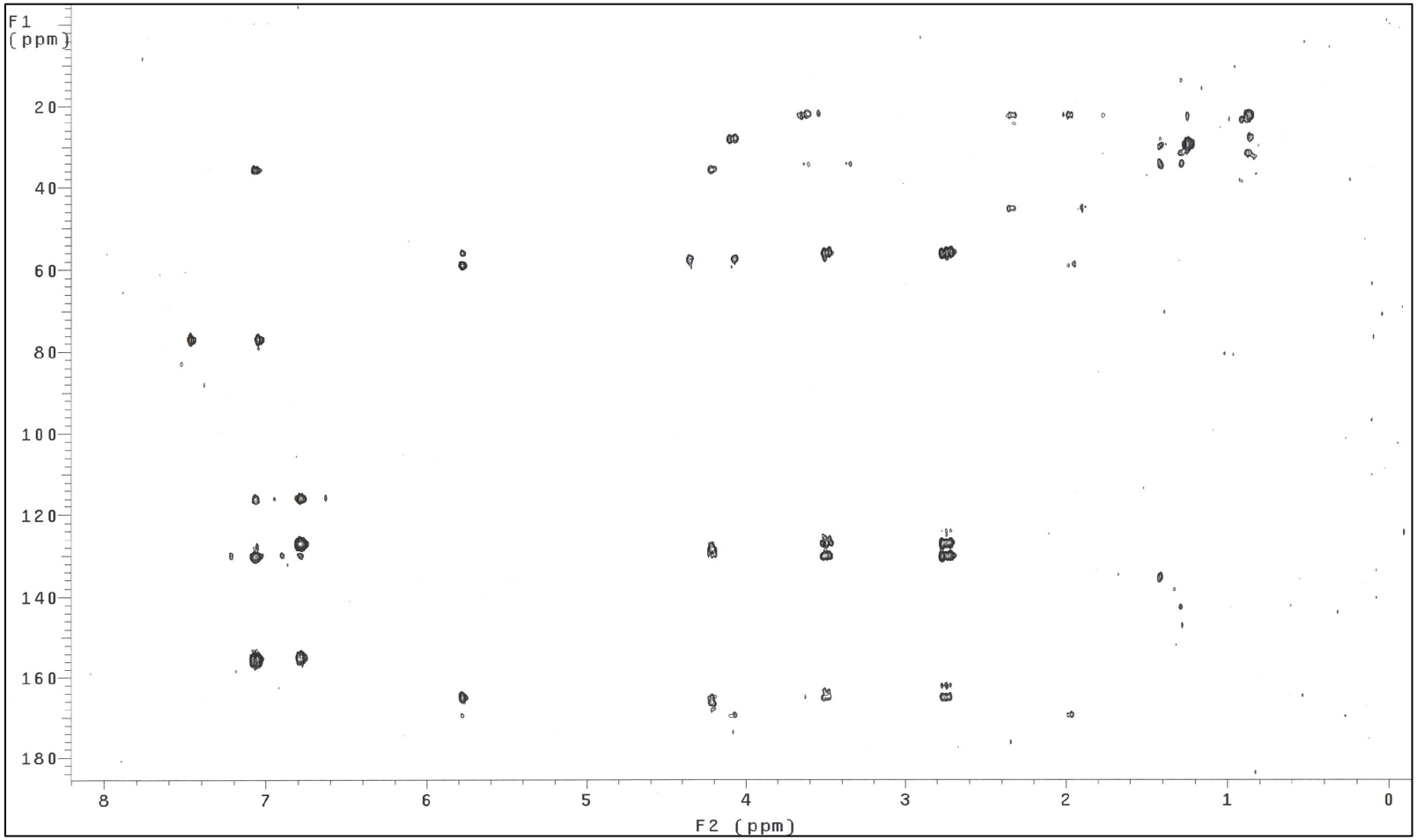
^{1}H NMR spectrum of compound **G5** (500 MHz, $CDCl_3$)



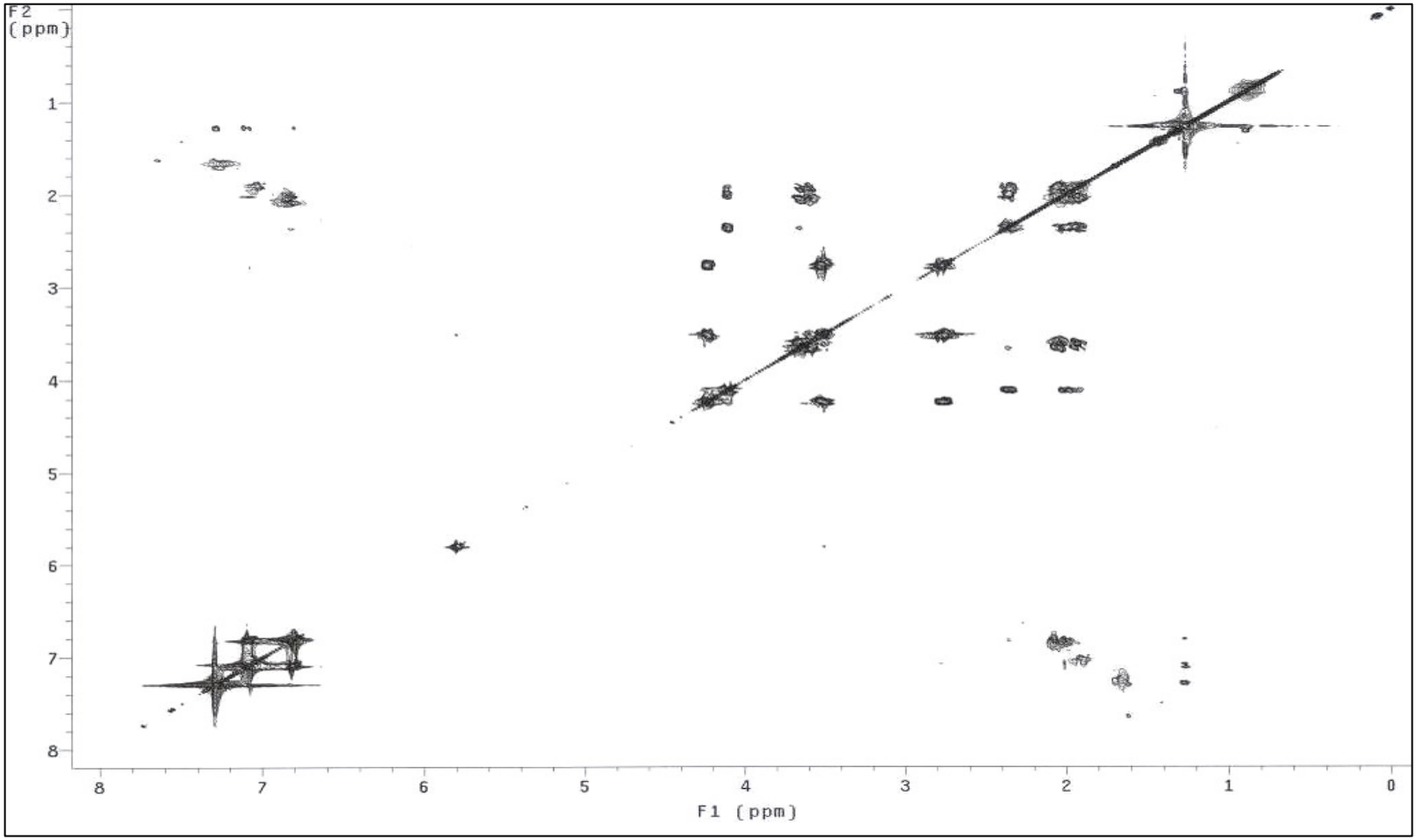
^{13}C NMR spectrum of compound **G5** (300 MHz, $CDCl_3$)



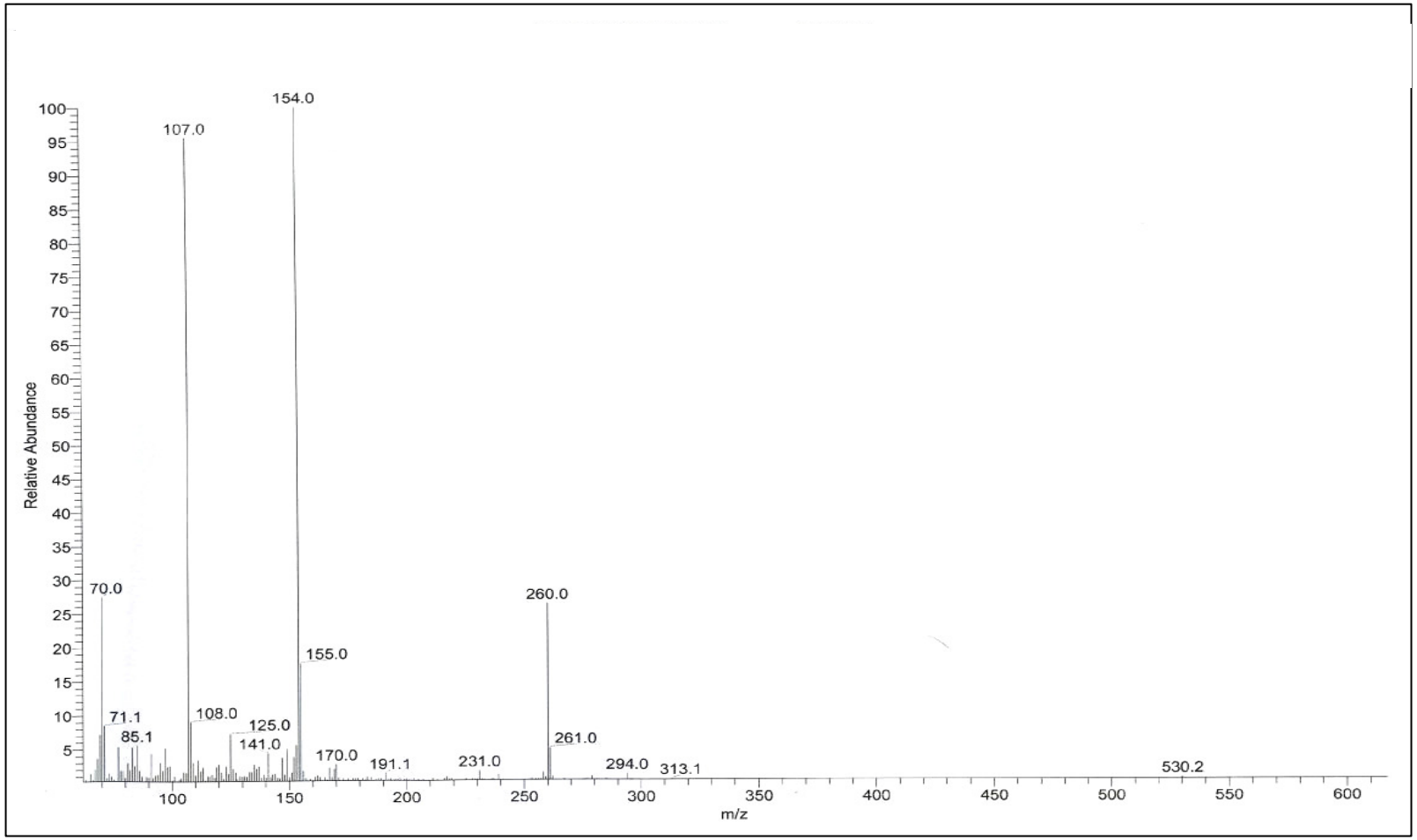
HMQC spectrum of compound **G5**



HMBC spectrum of compound **G5**

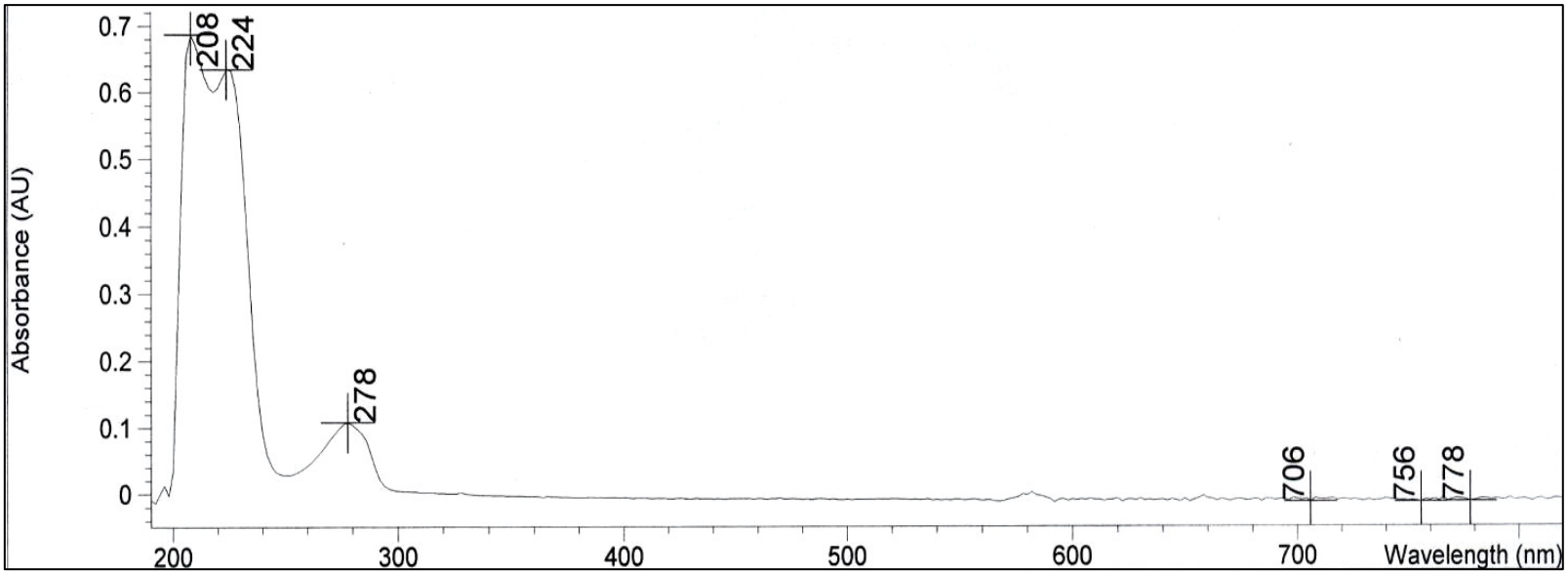


COSY spectrum of compound **G5**

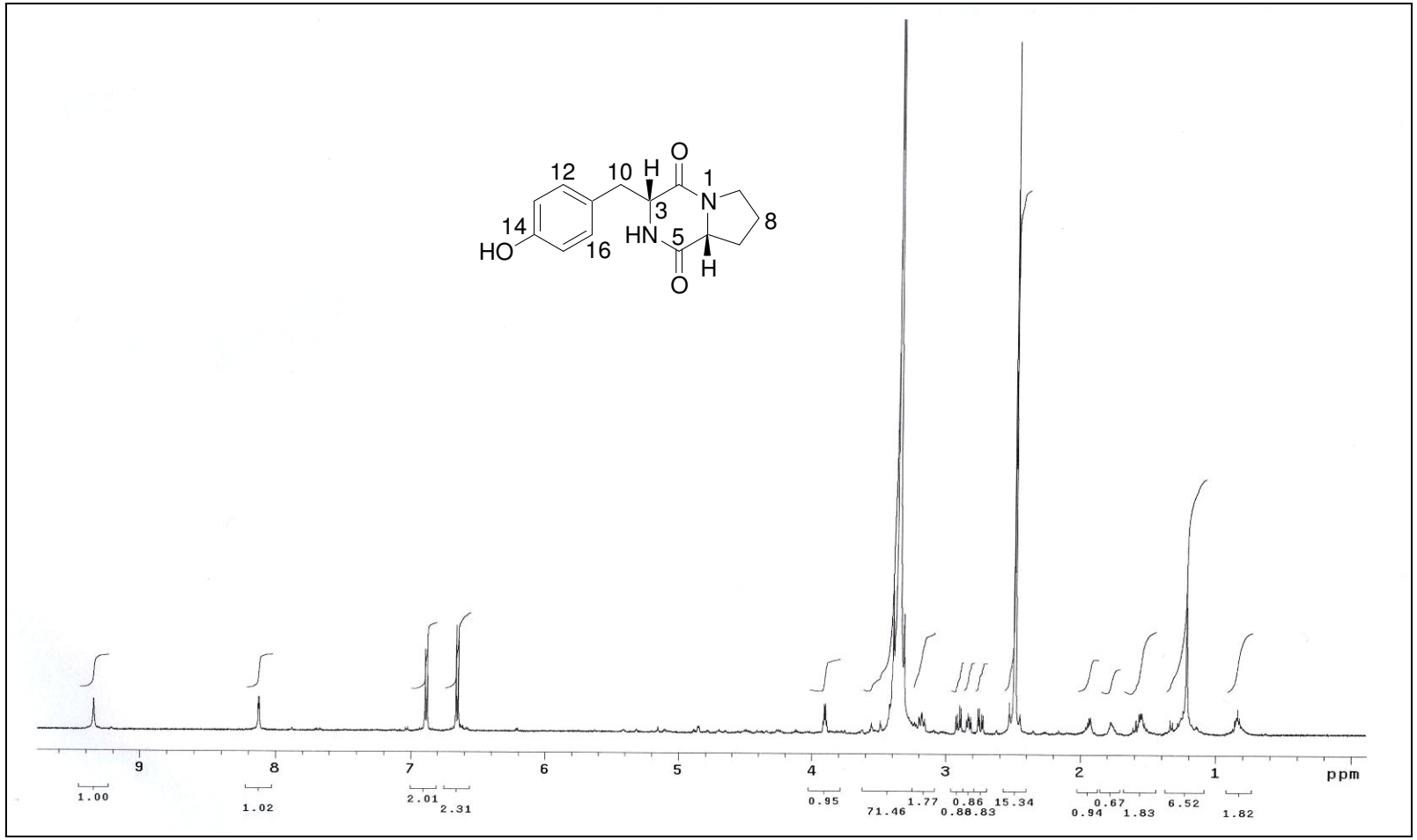


EI spectrum of compound **G5**

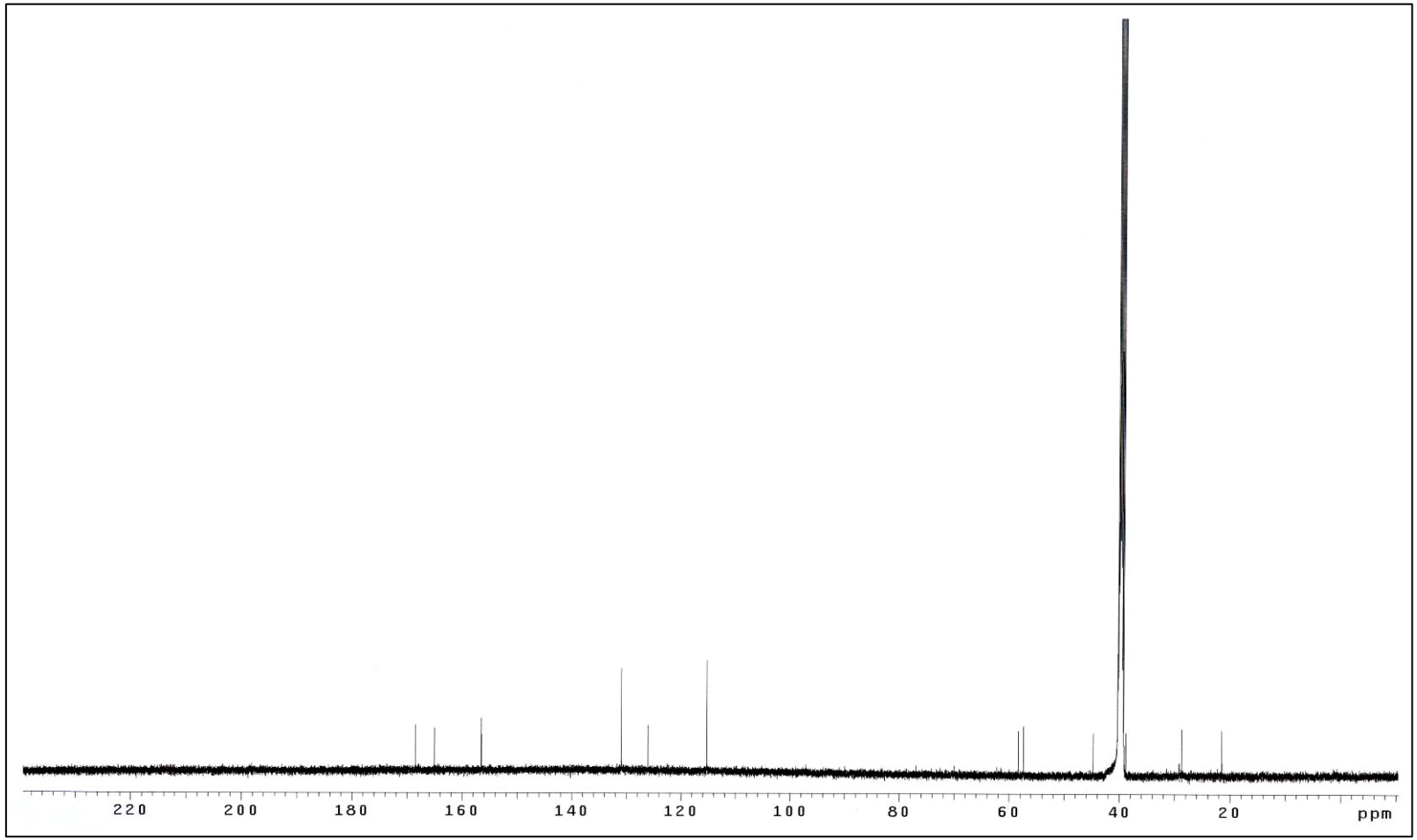
IR spectrum of compound **G5**



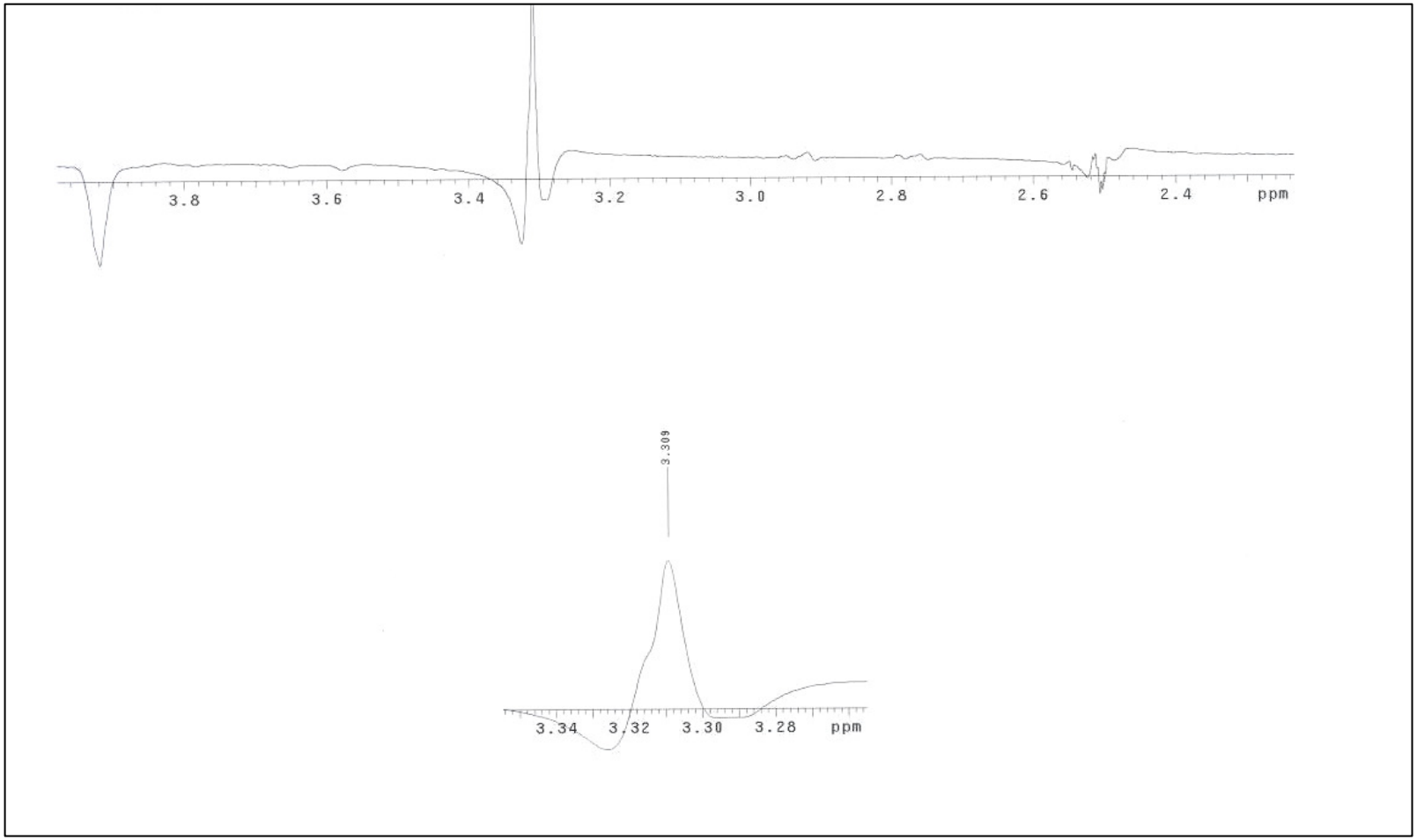
UV spectrum of compound **G5**



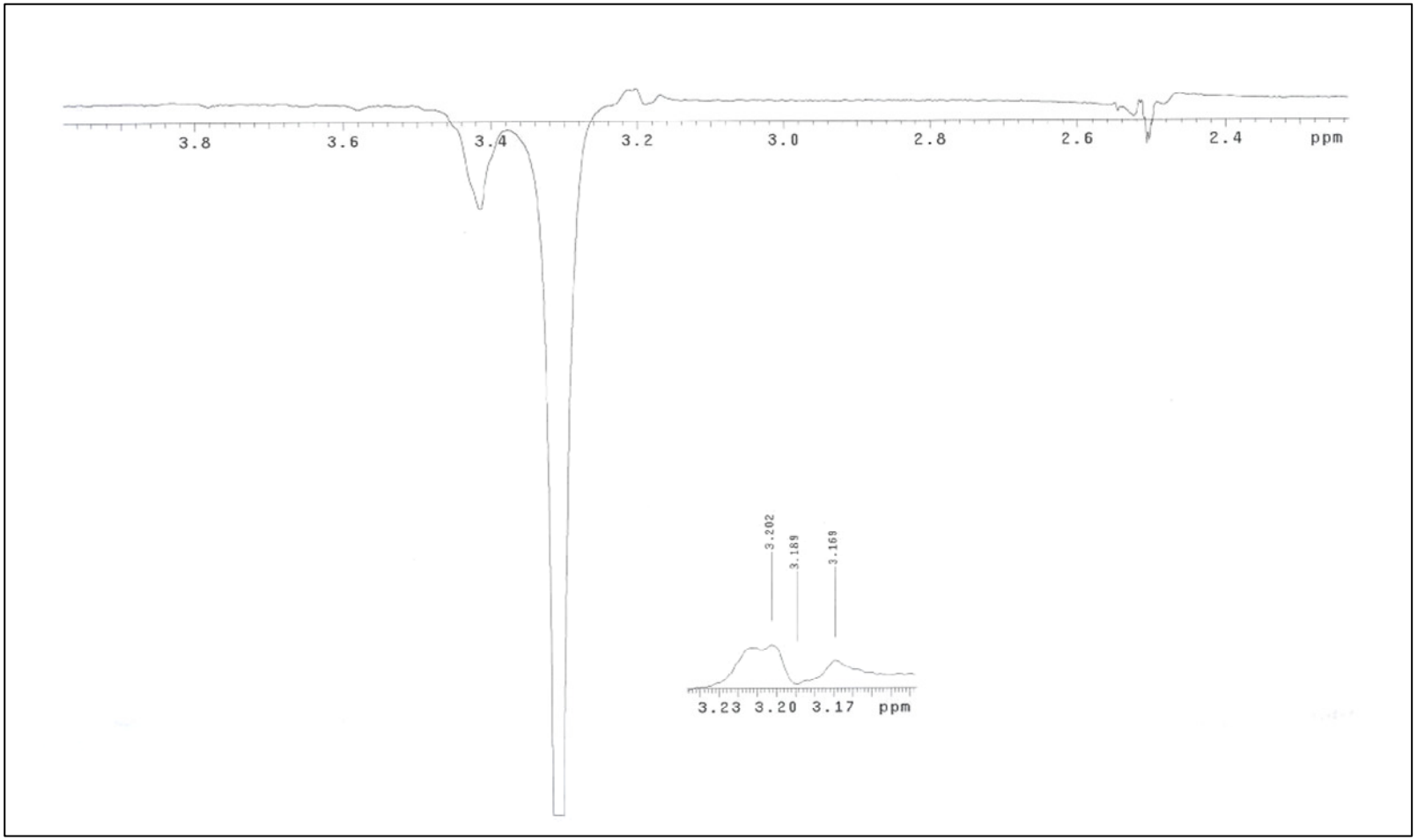
^{1}H NMR spectrum of compound **G6** (500 MHz, DMSO-d_6)



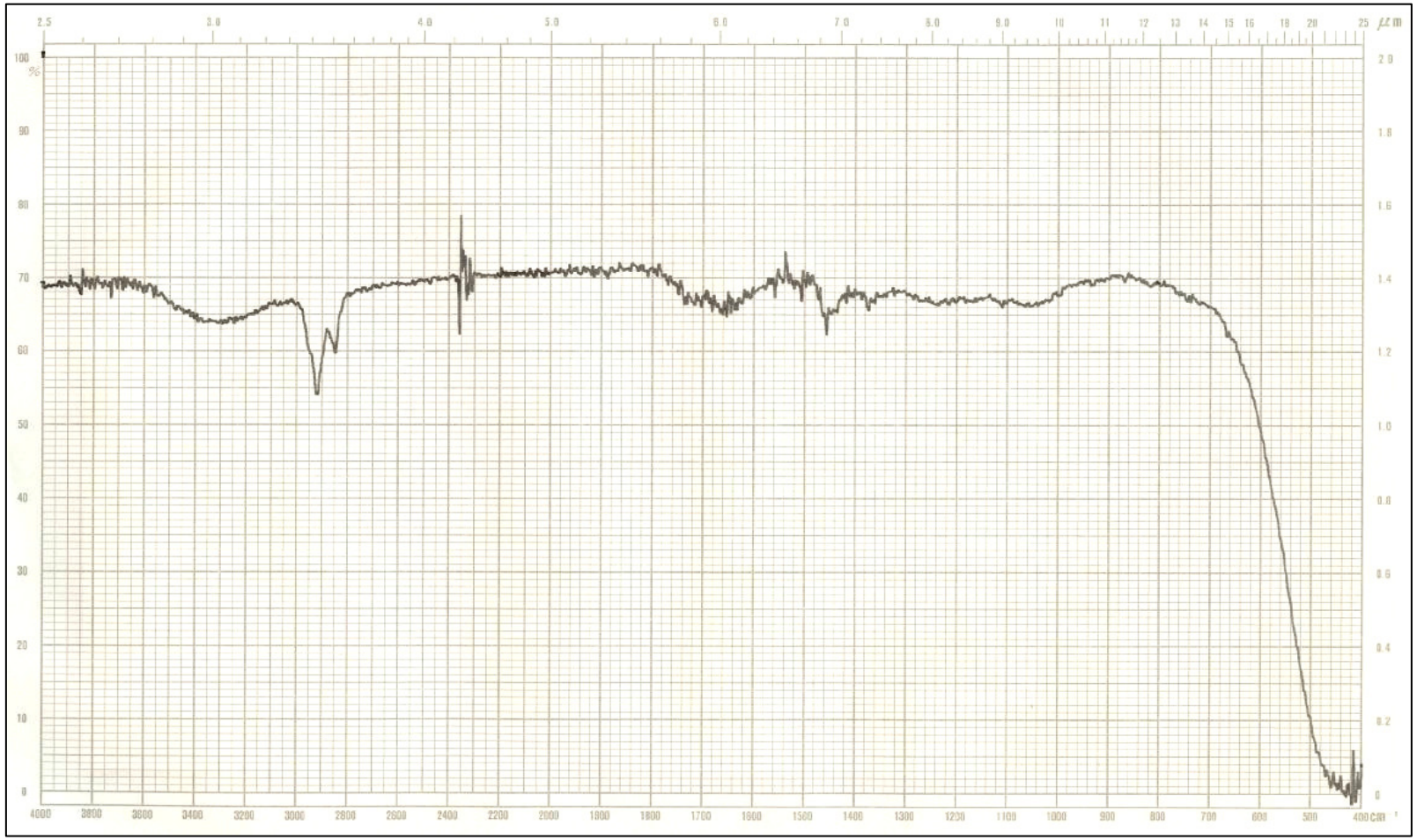
^{13}C NMR spectrum of compound **G6** (300 MHz, DMSO-d_6)



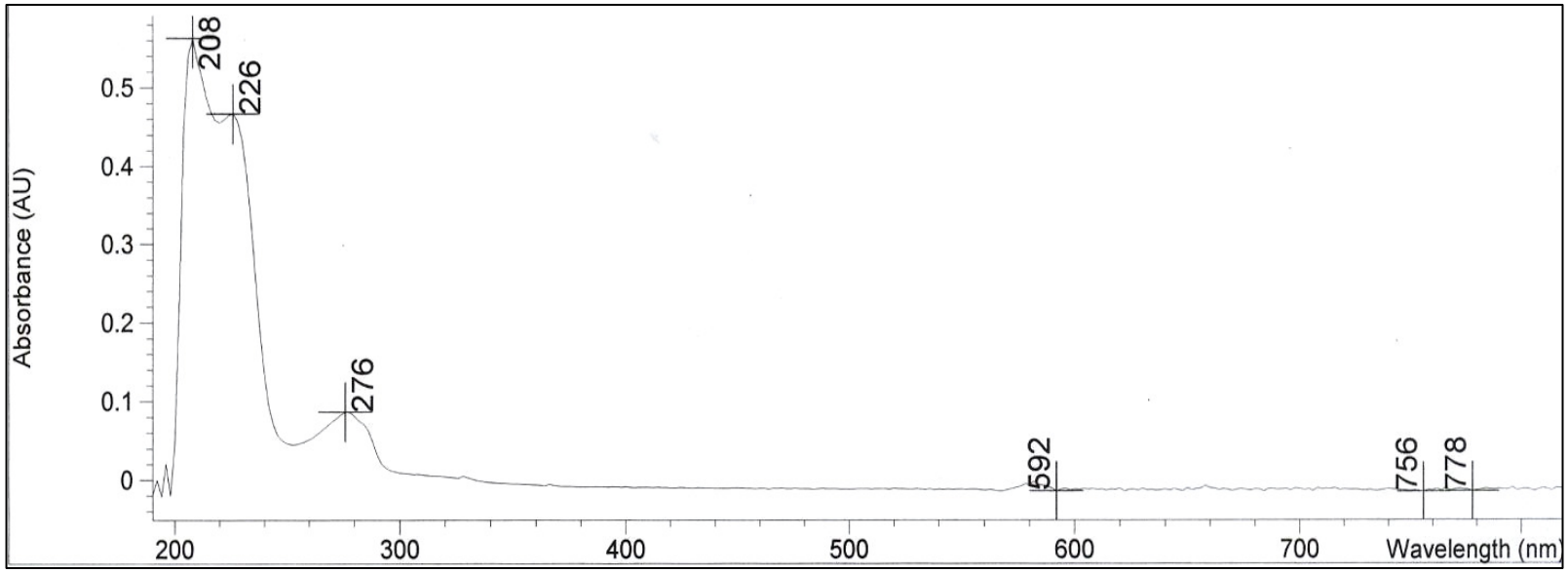
NOE spectrum of compound **G6**



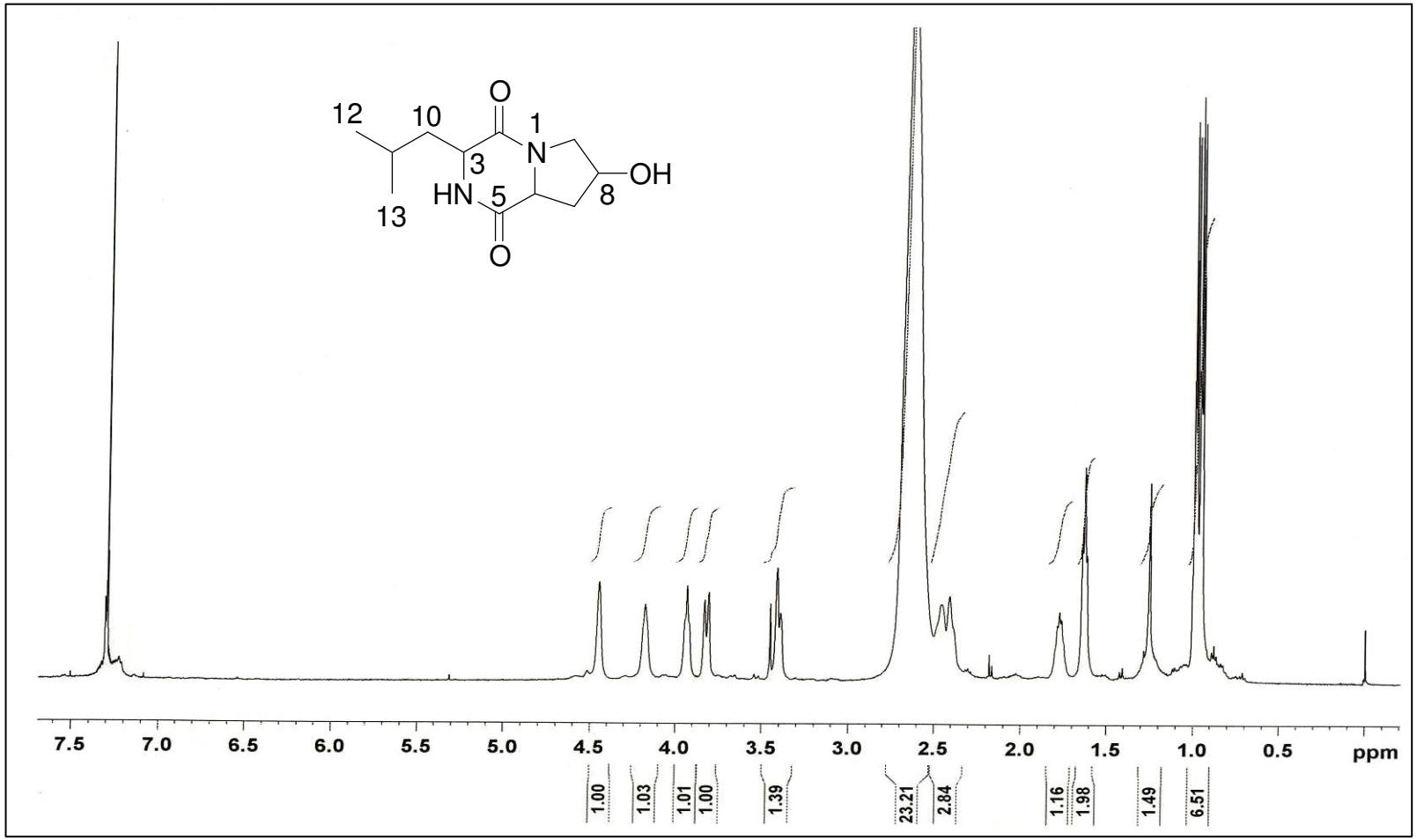
NOE spectrum of compound **G6**



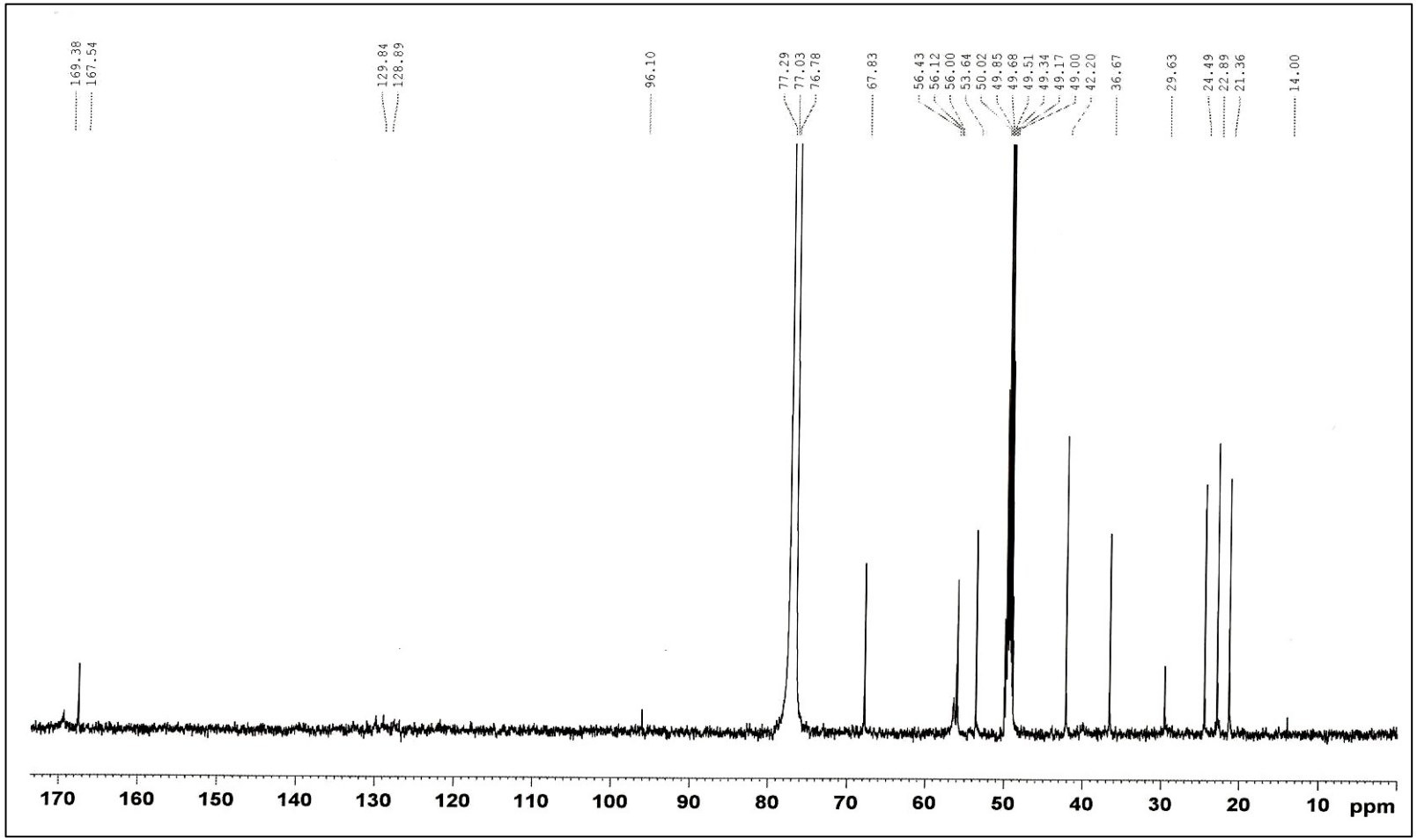
IR spectrum of compound **G6**



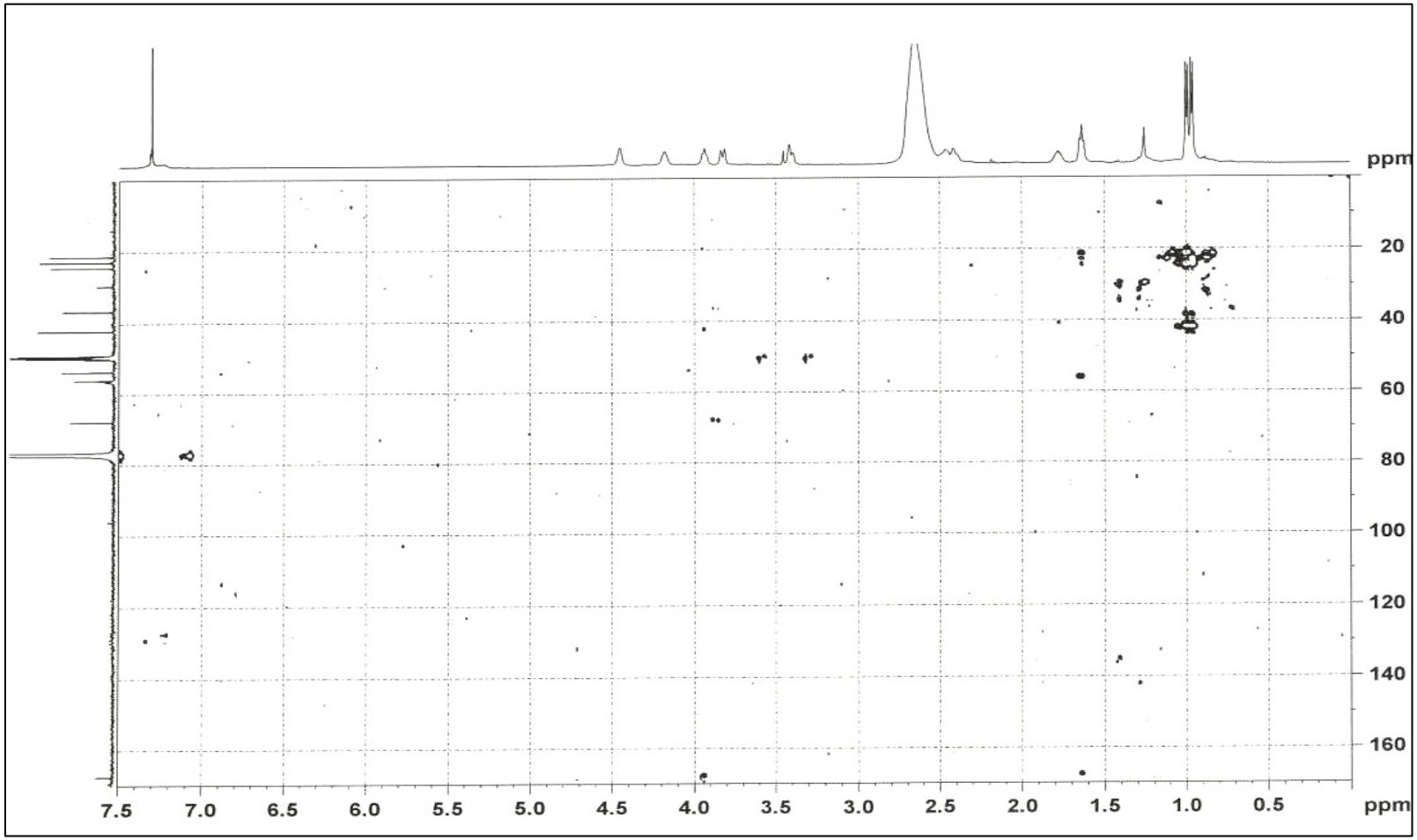
UV spectrum of compound **G6**



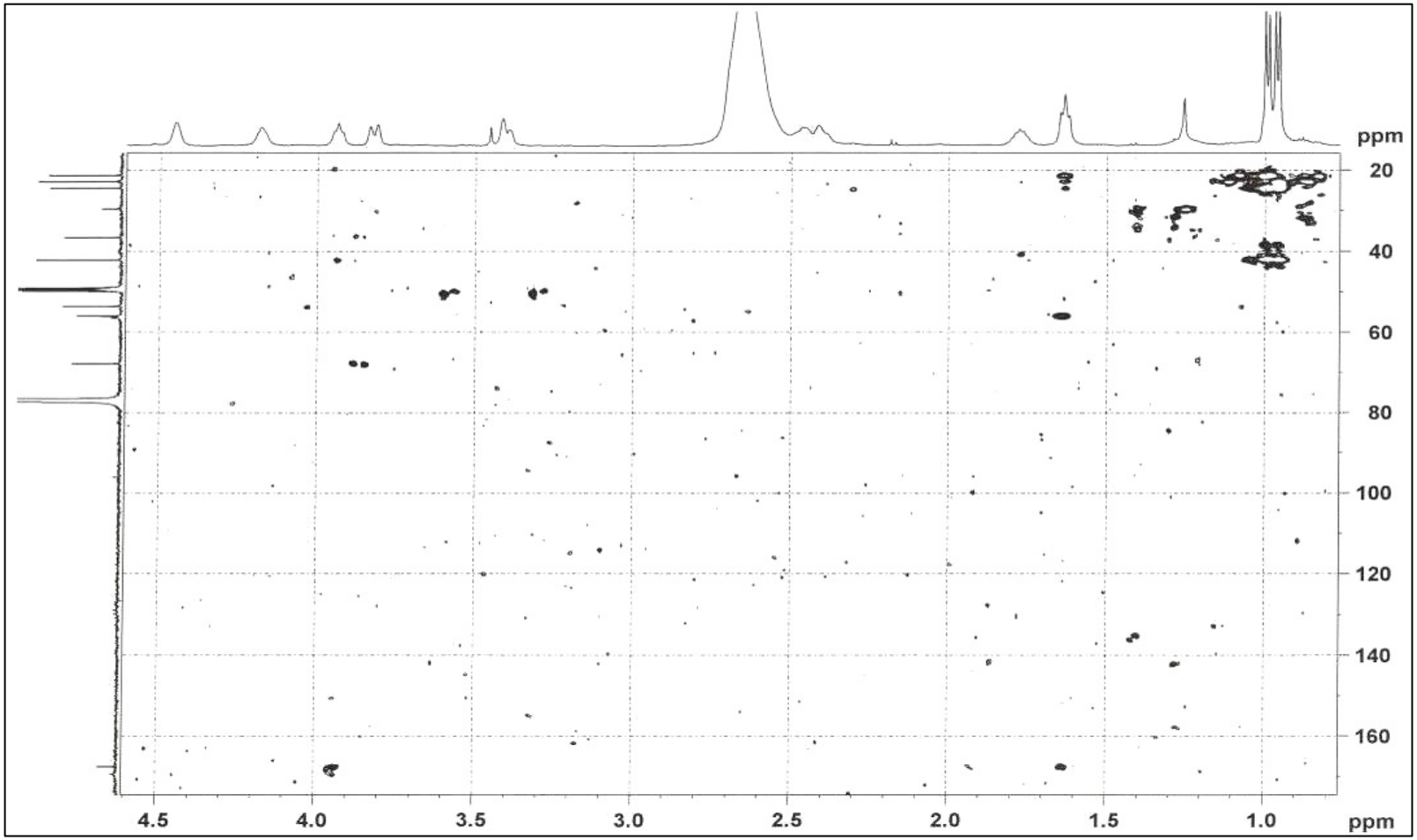
^{1}H NMR spectrum of compound **G7** (300 MHz, $CDCl_3$+CD_3OD)



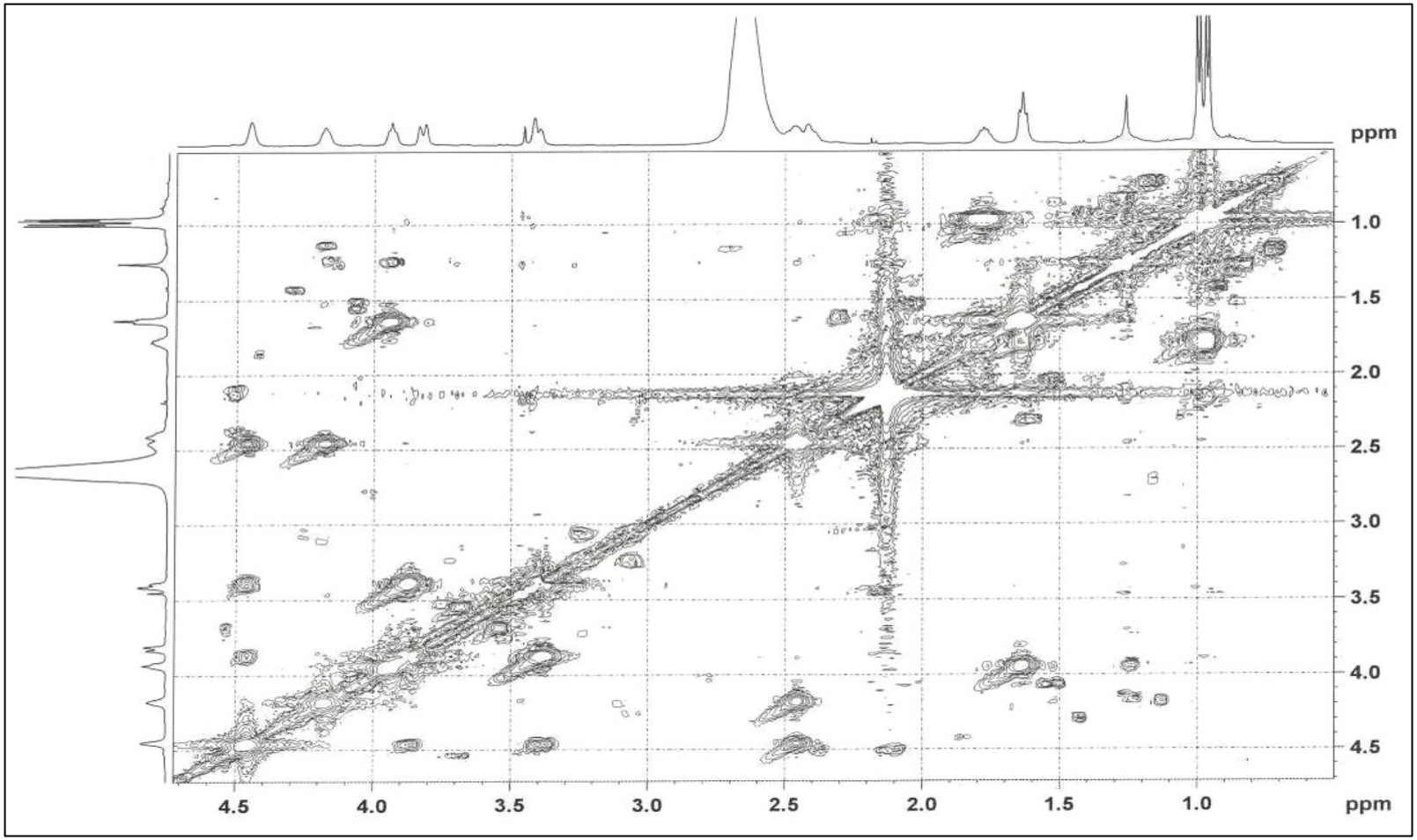
^{13}C NMR spectrum of compound **G7** (300 MHz, $CDCl_3$+CD_3OD)



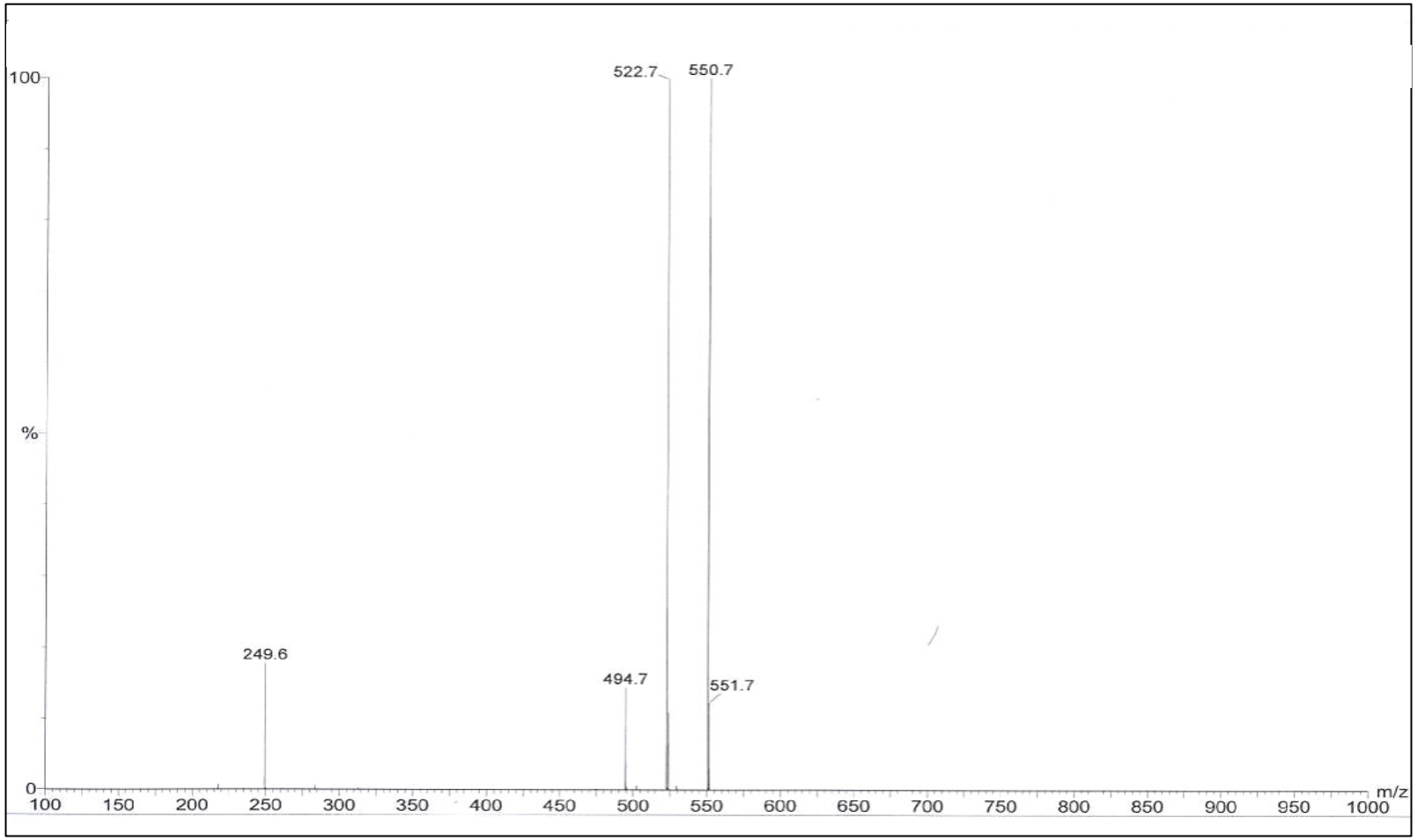
HMQC spectrum of compound **G7**



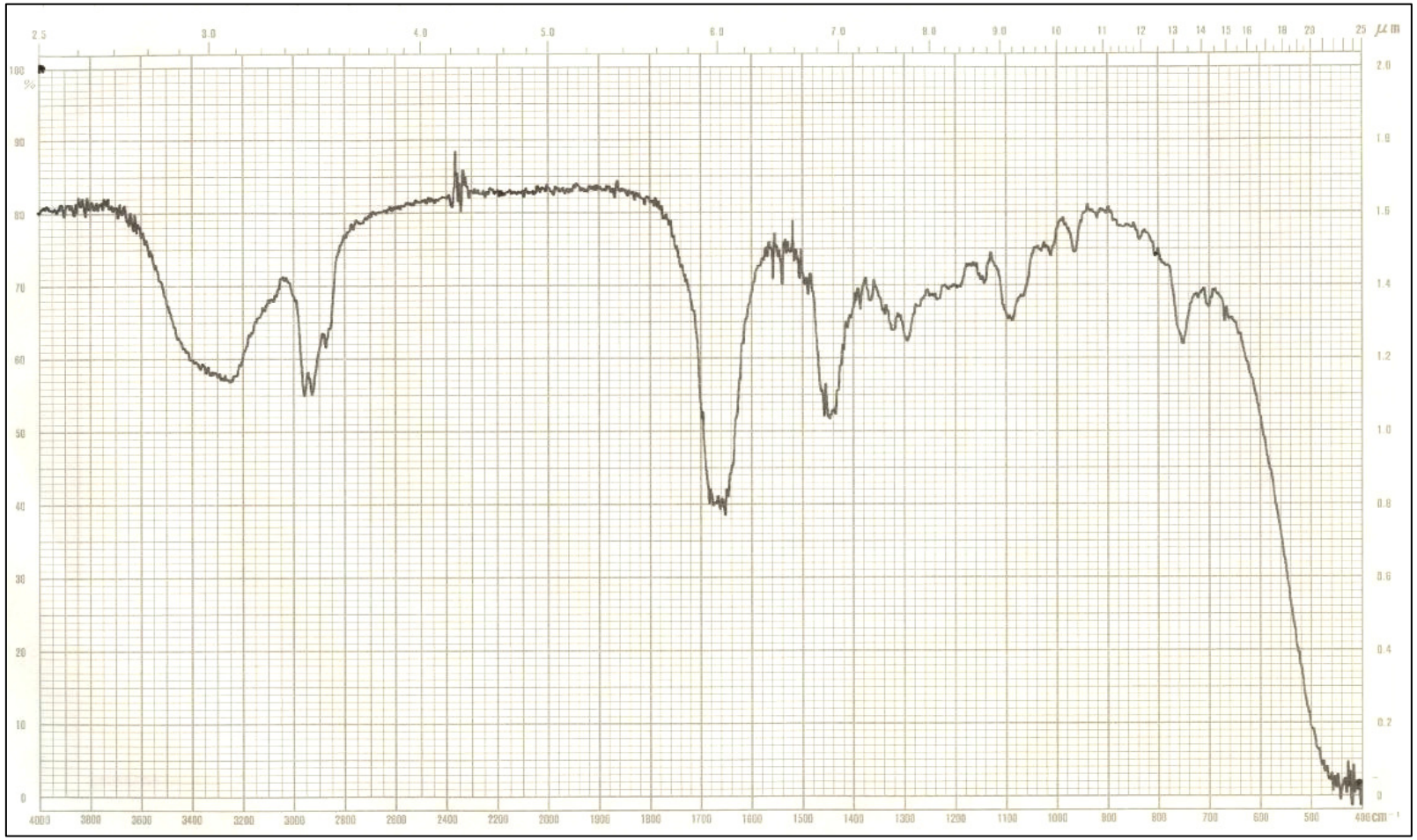
HMBC spectrum of compound **G7**



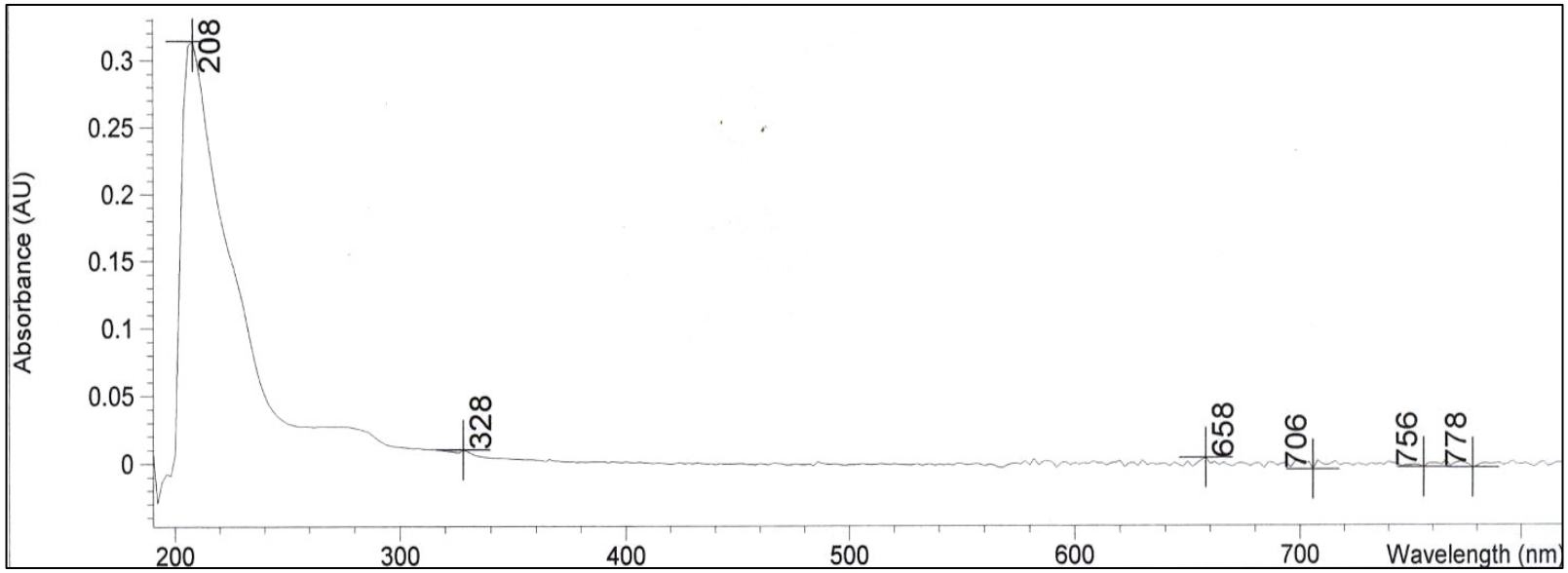
COSY spectrum of compound **G7**



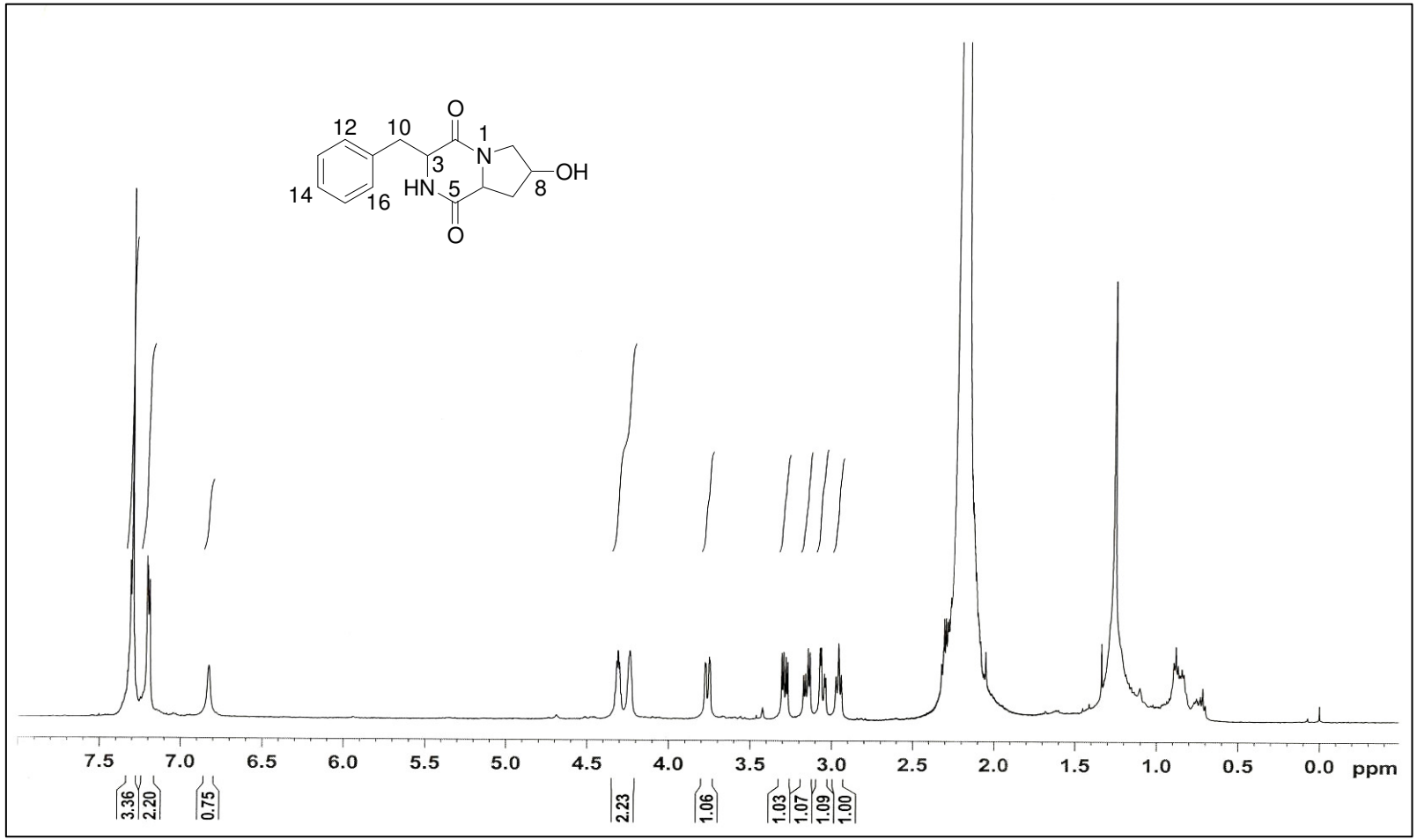
ESIMS spectrum of compound **G7**



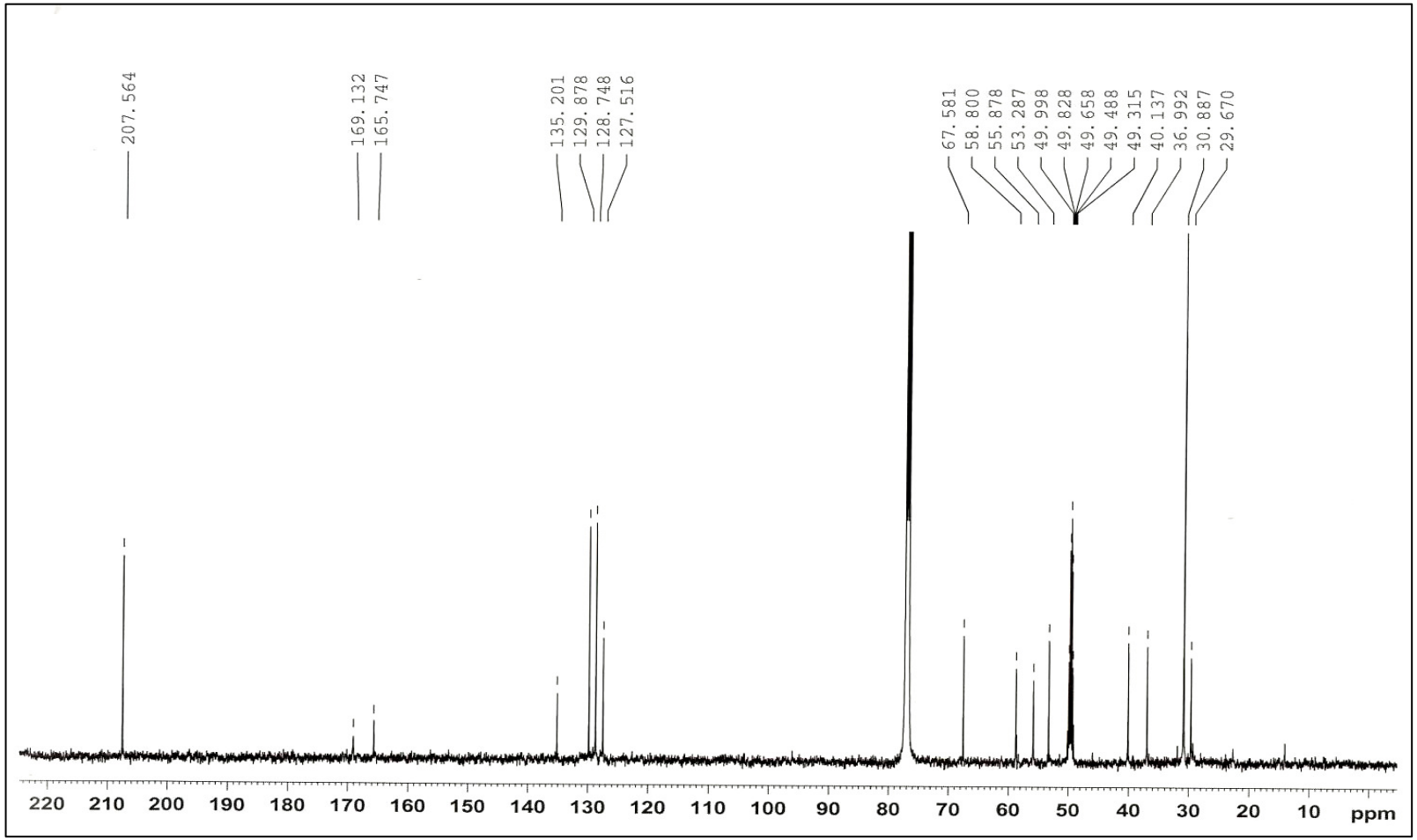
IR spectrum of compound **G7**



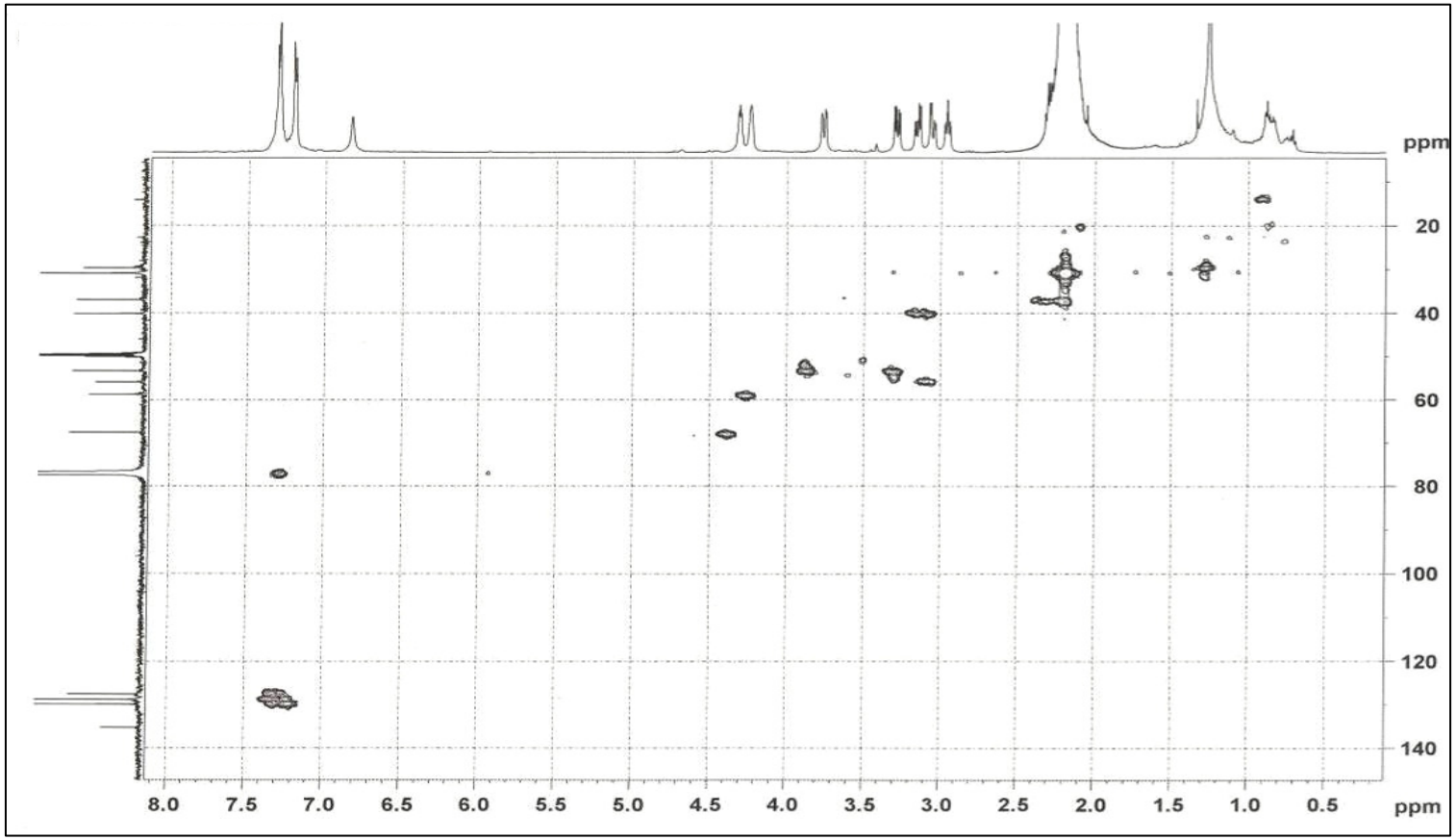
UV spectrum of compound **G7**



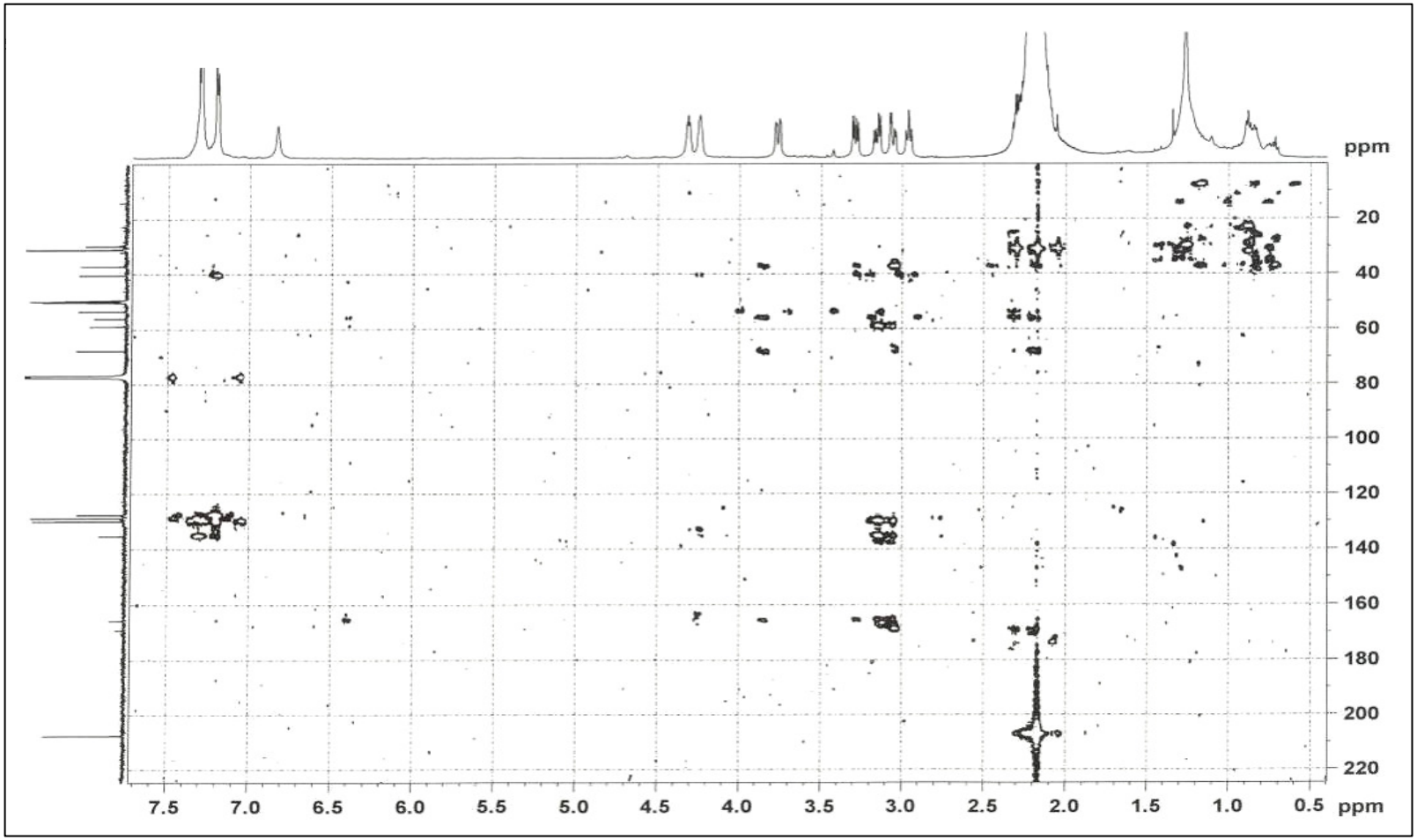
^{1}H NMR spectrum of compound **G8** (300 MHz, $CDCl_3$+CD_3OD)



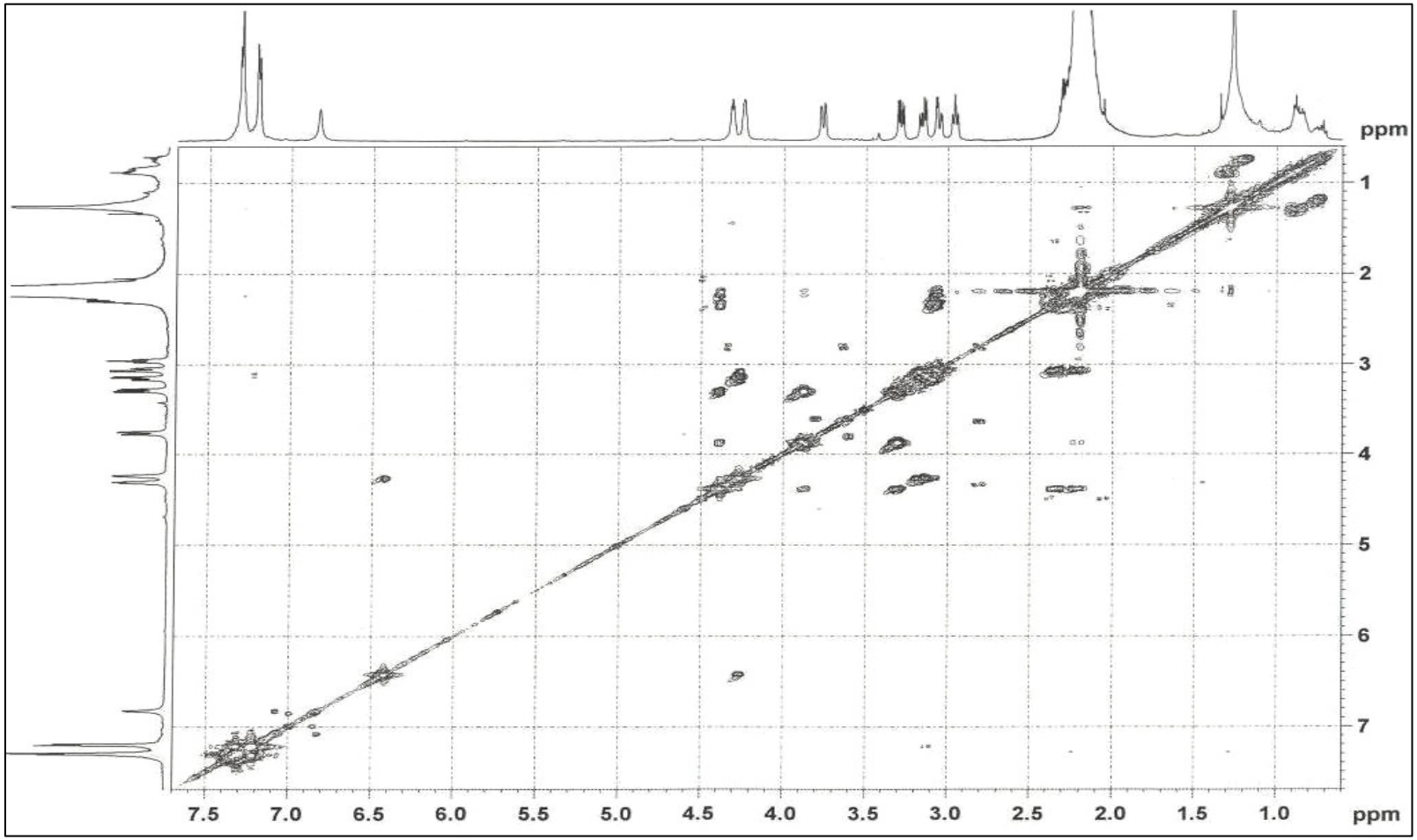
^{13}C NMR spectrum of compound **G8** (300 MHz, $CDCl_3$+CD_3OD)



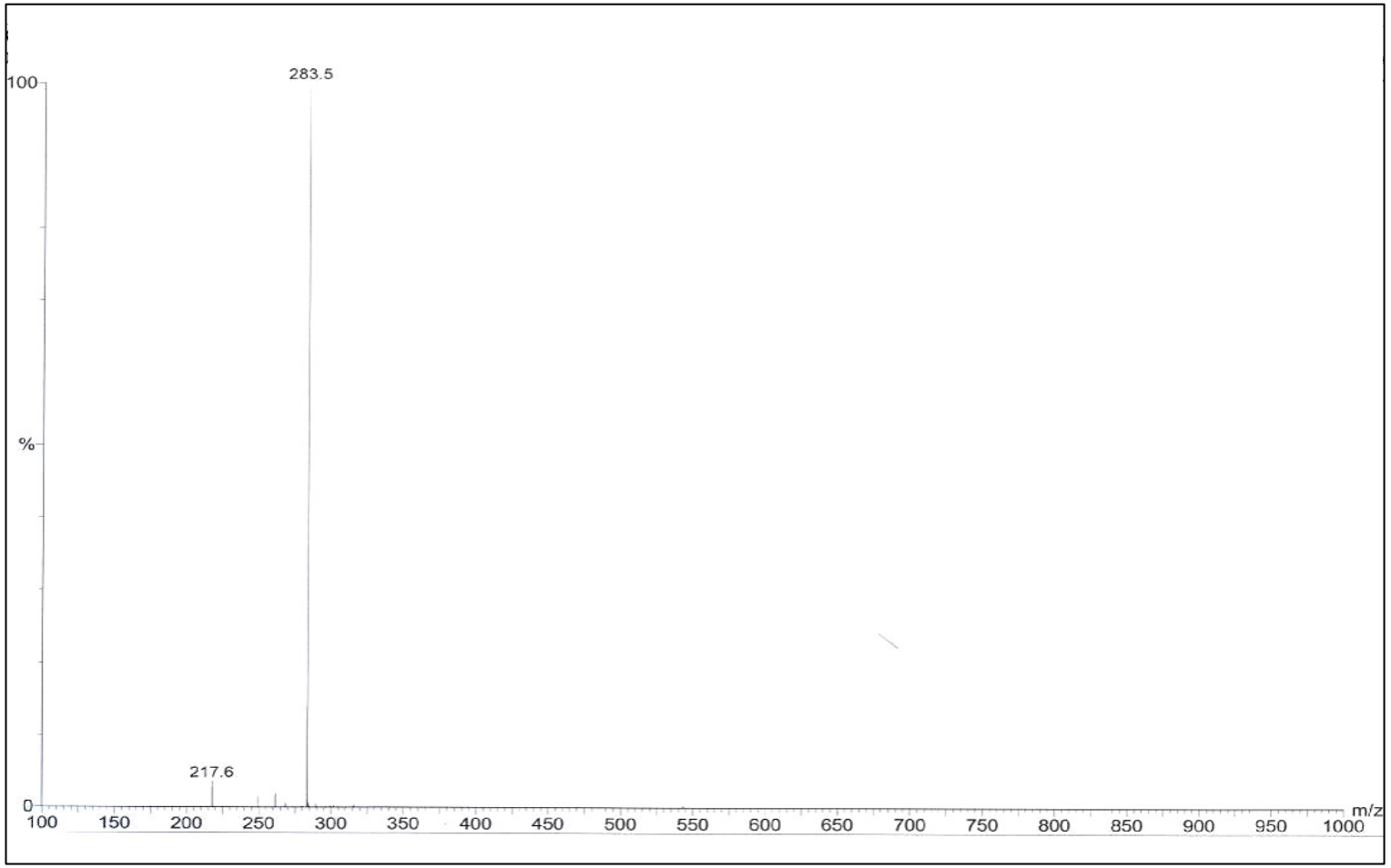
HMQC spectrum of compound **G8**



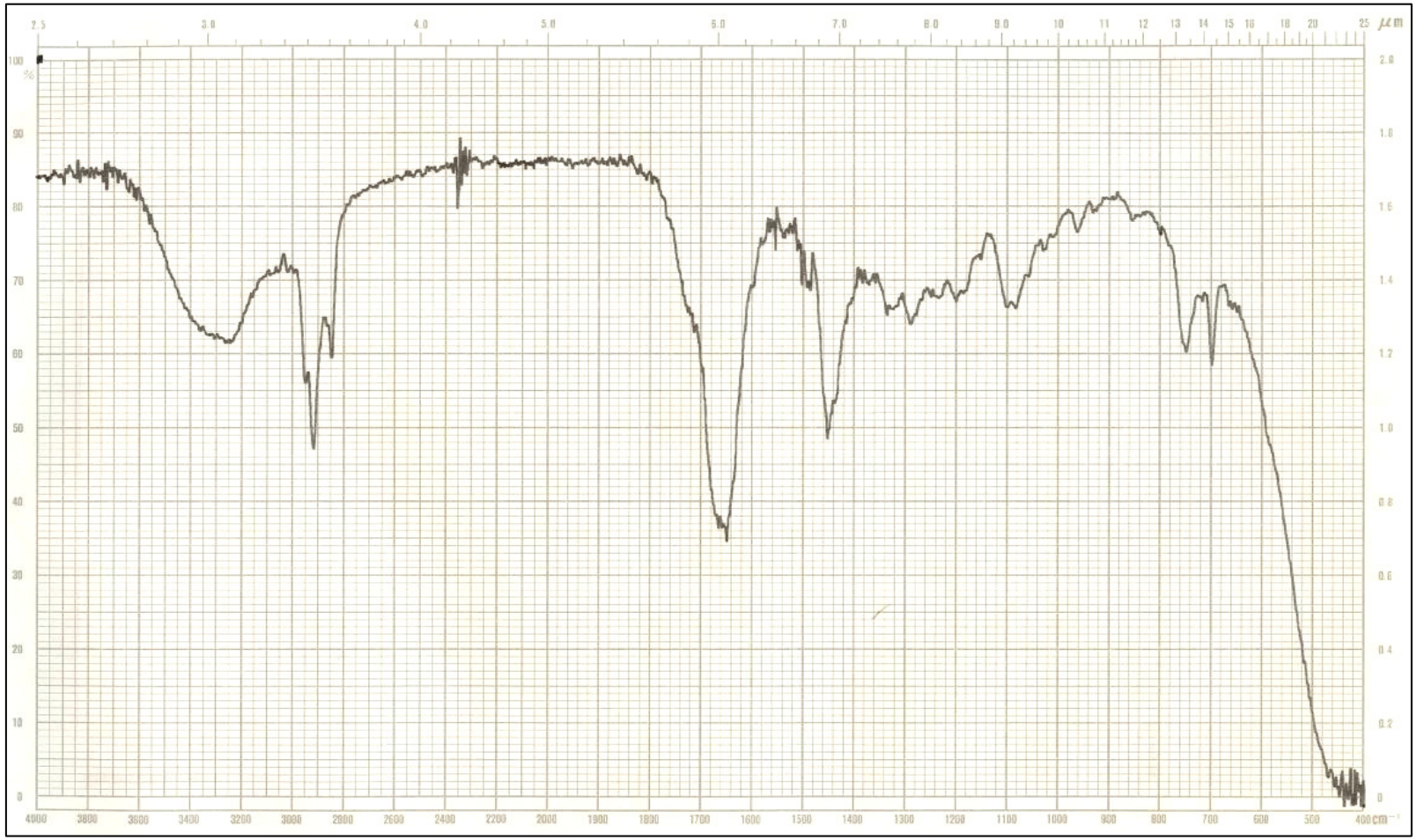
HMBC spectrum of compound **G8**



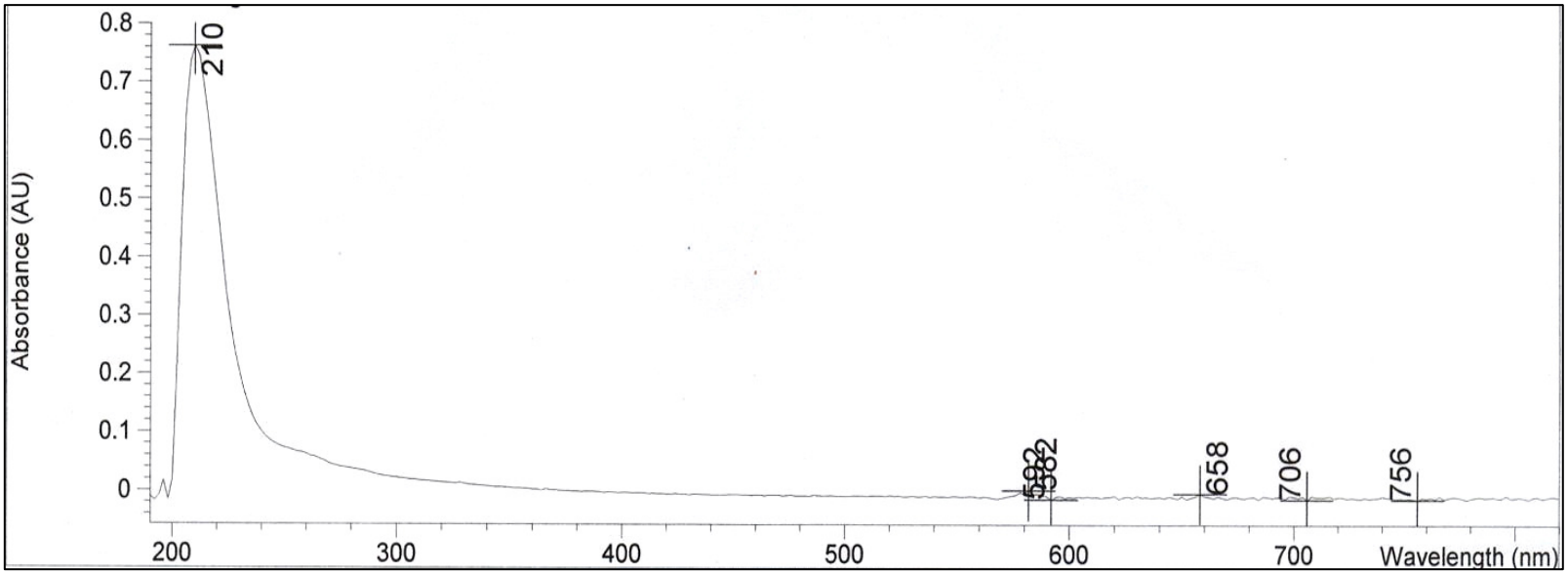
COSY spectrum of compound **G8**



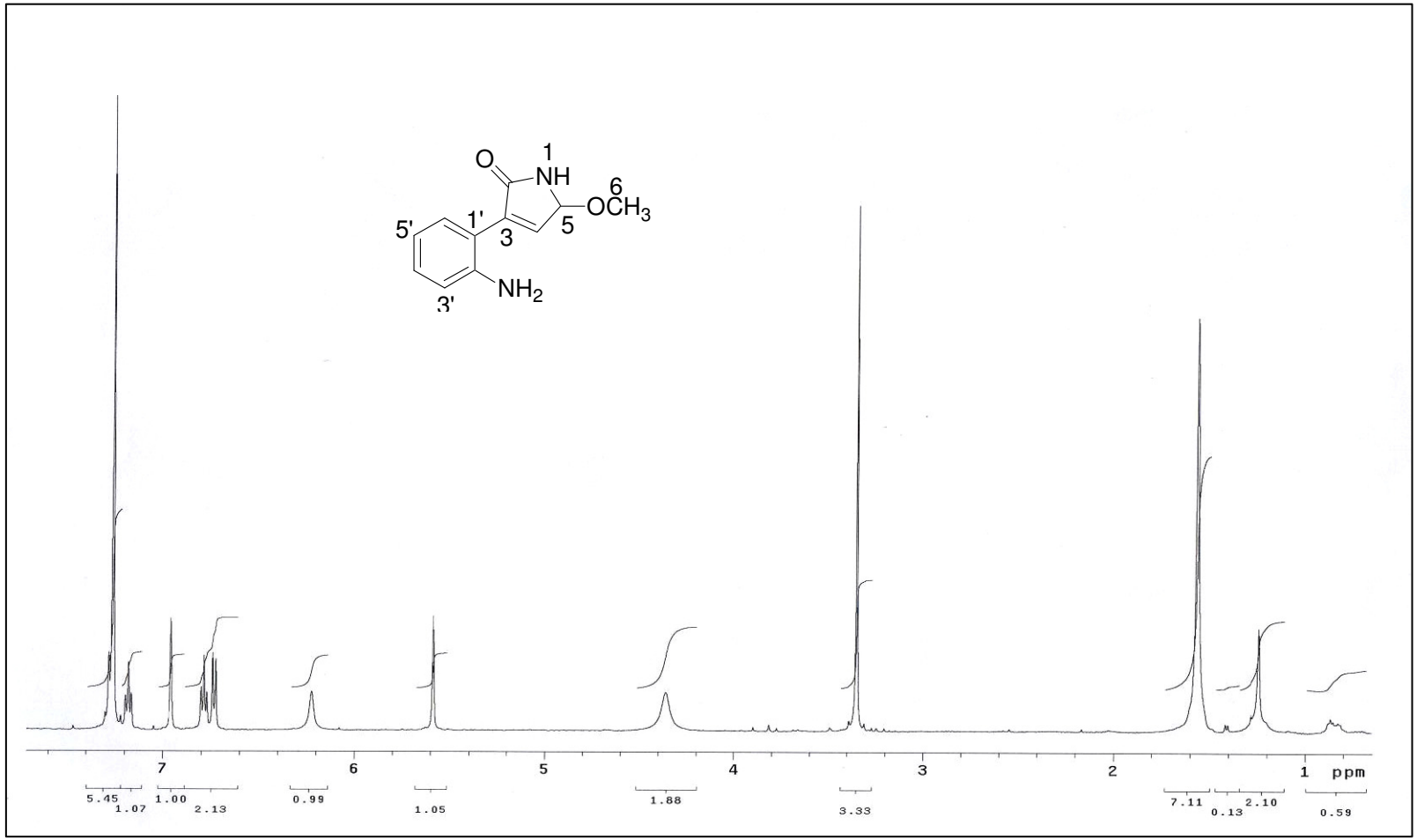
ESIMS spectrum of compound **G8**



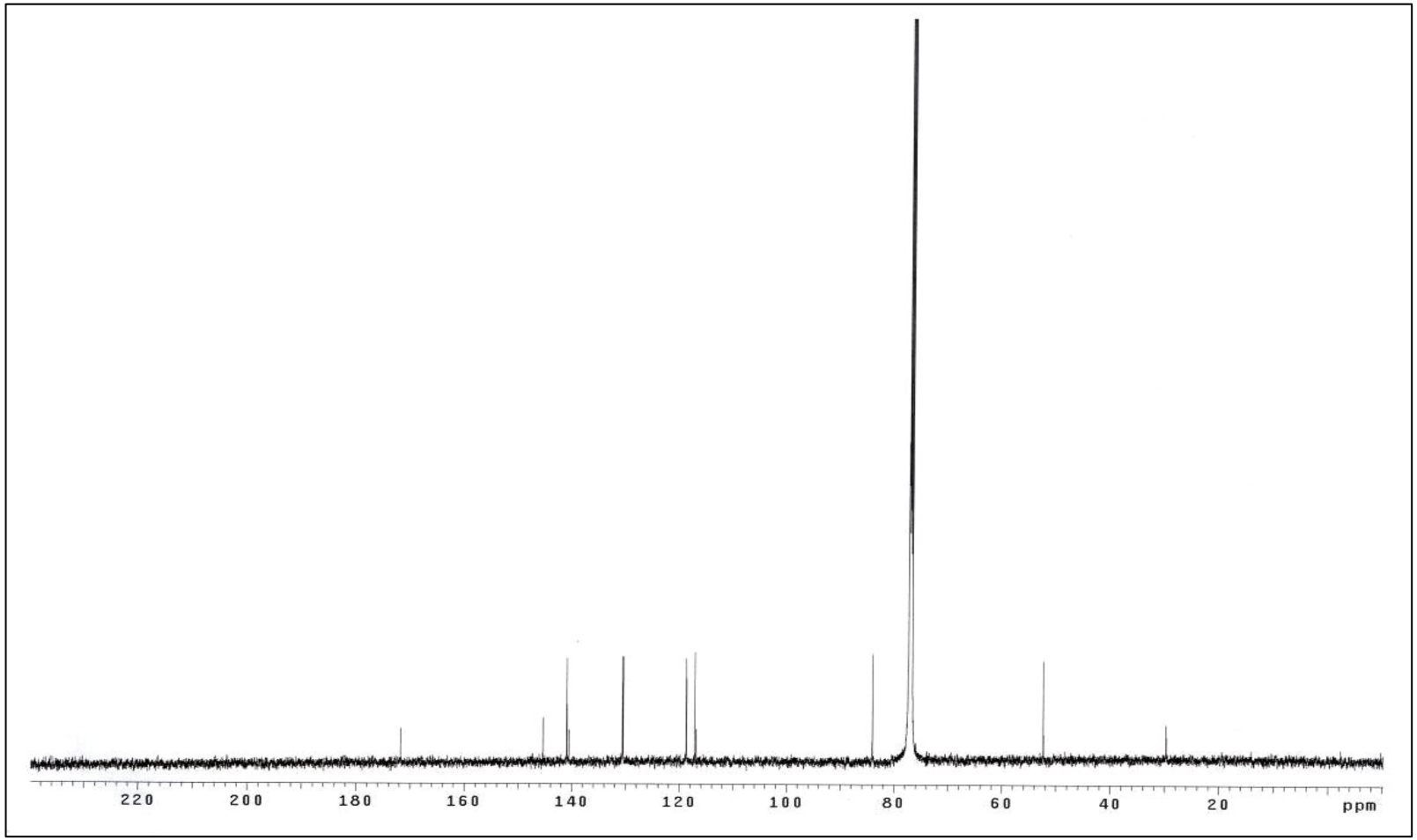
IR spectrum of compound **G8**



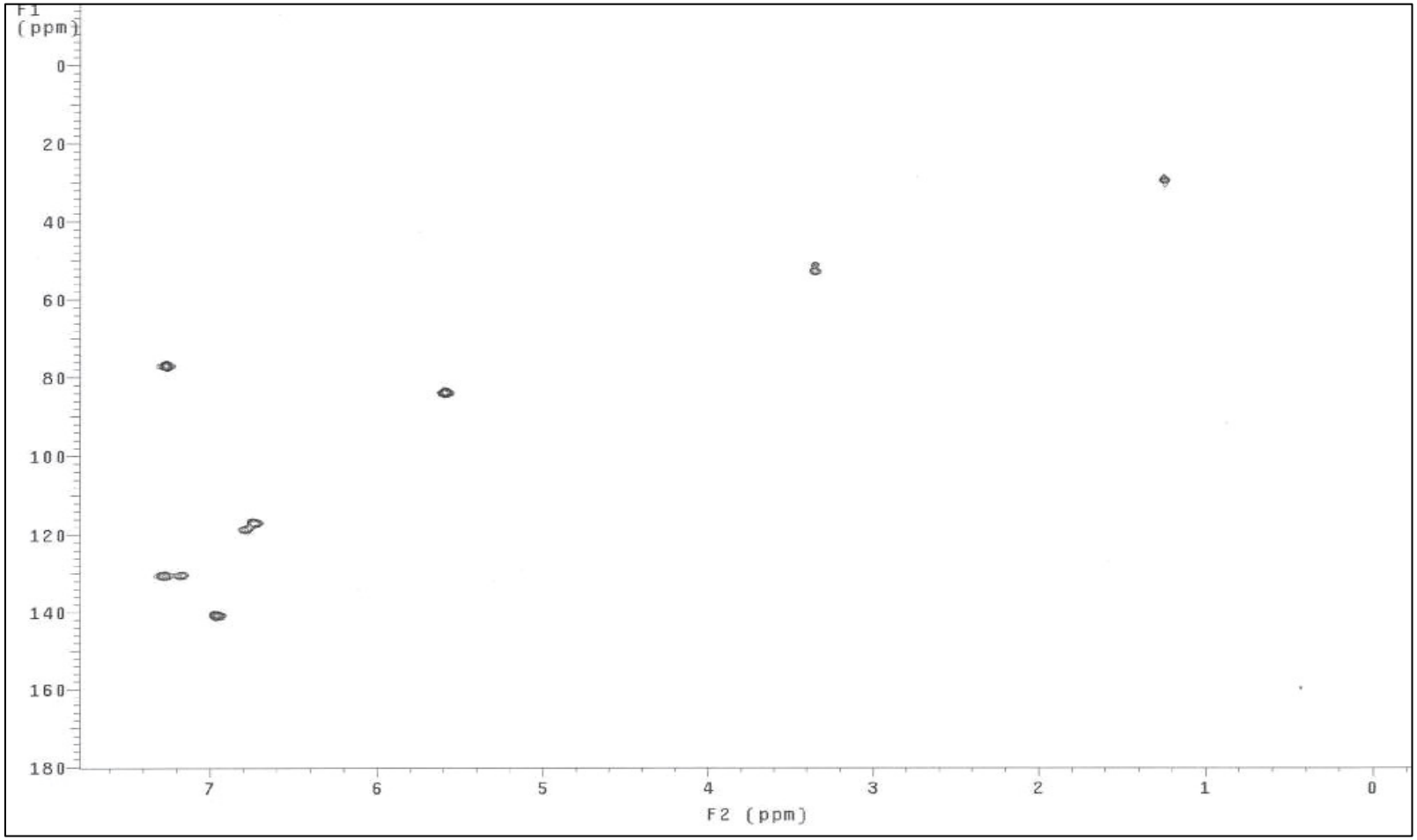
UV spectrum of compound **G8**



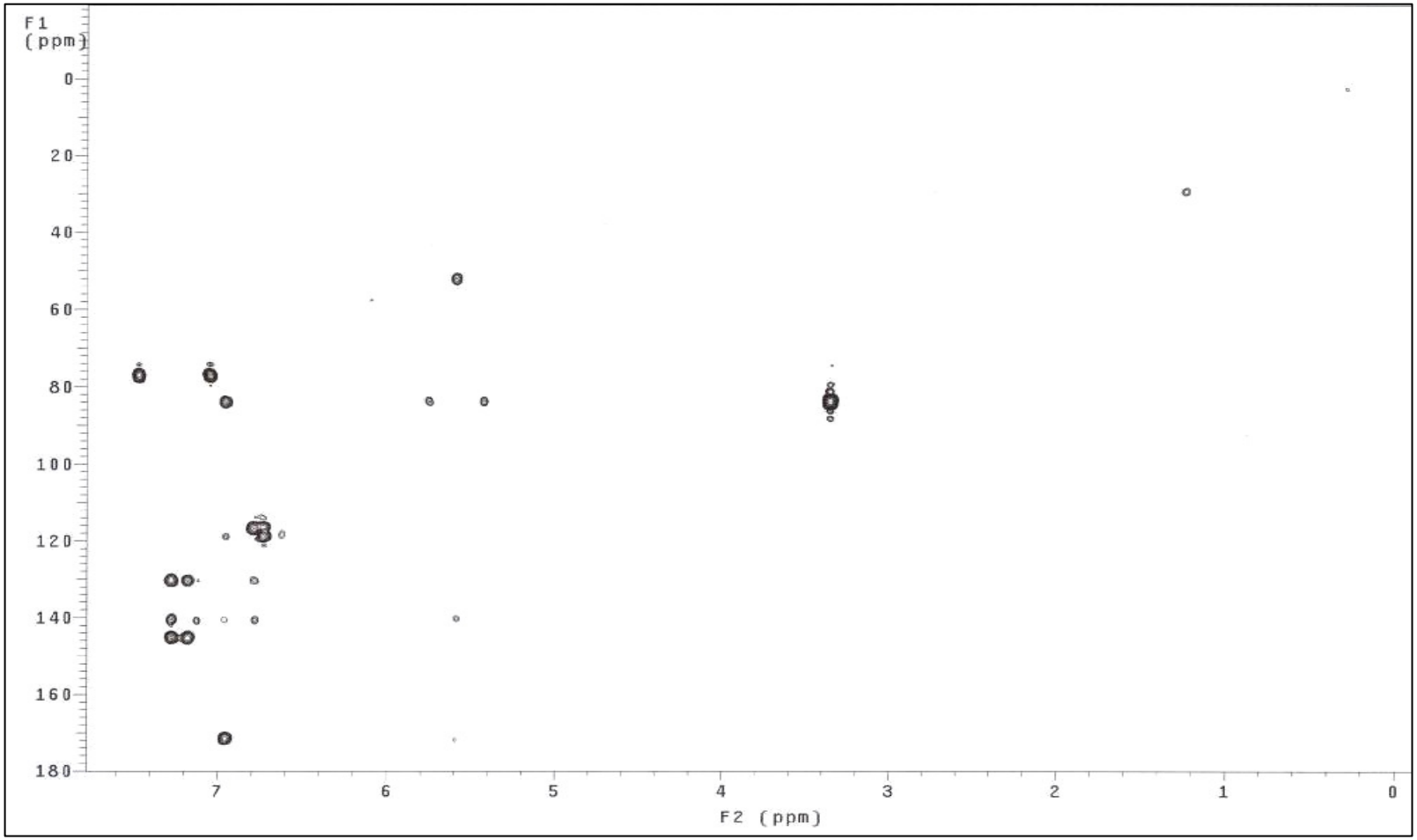
^{1}H NMR spectrum of compound **G9** (500 MHz, $CDCl_3$)



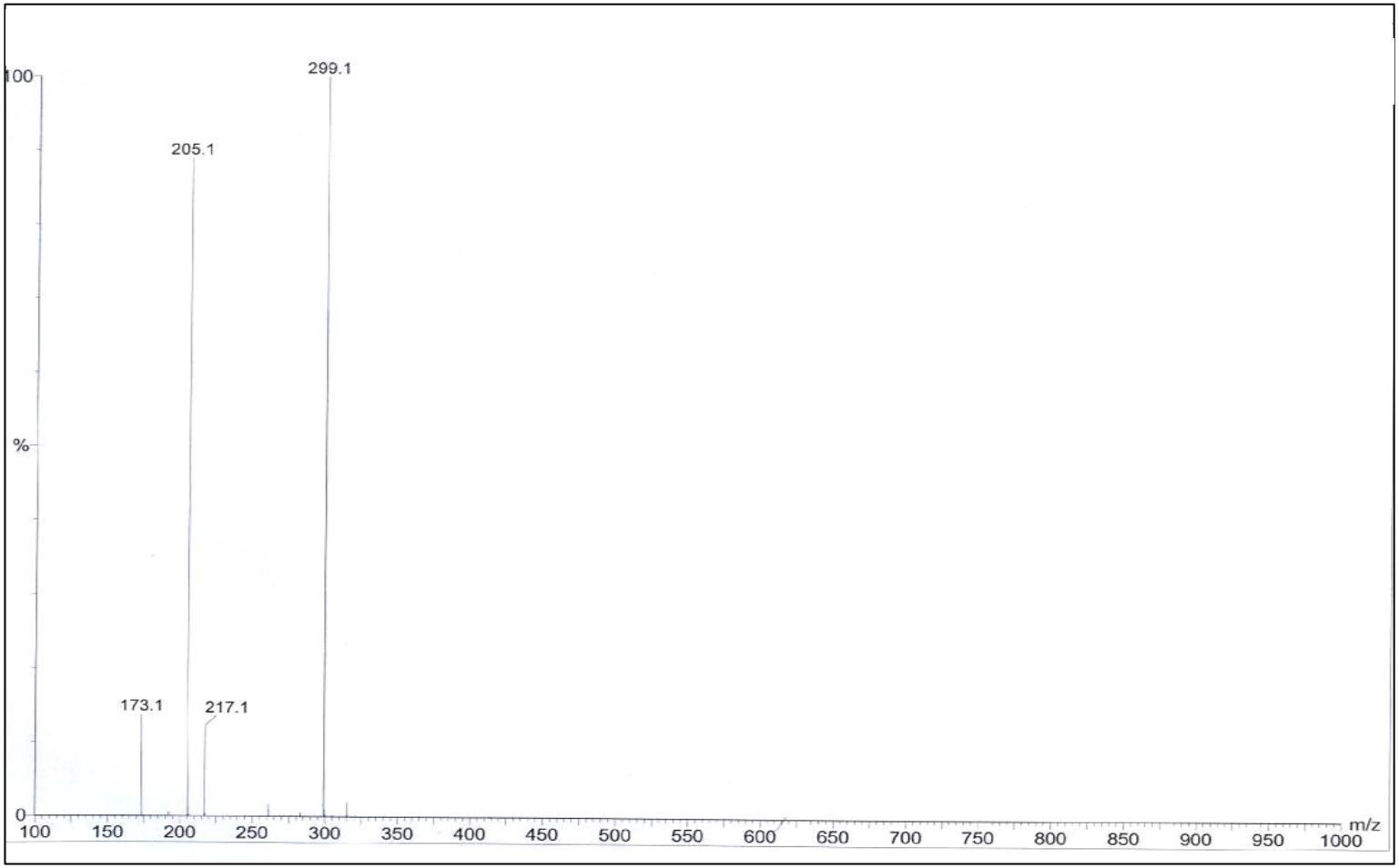
^{13}C NMR spectrum of compound **G9** (300 MHz, $CDCl_3$)



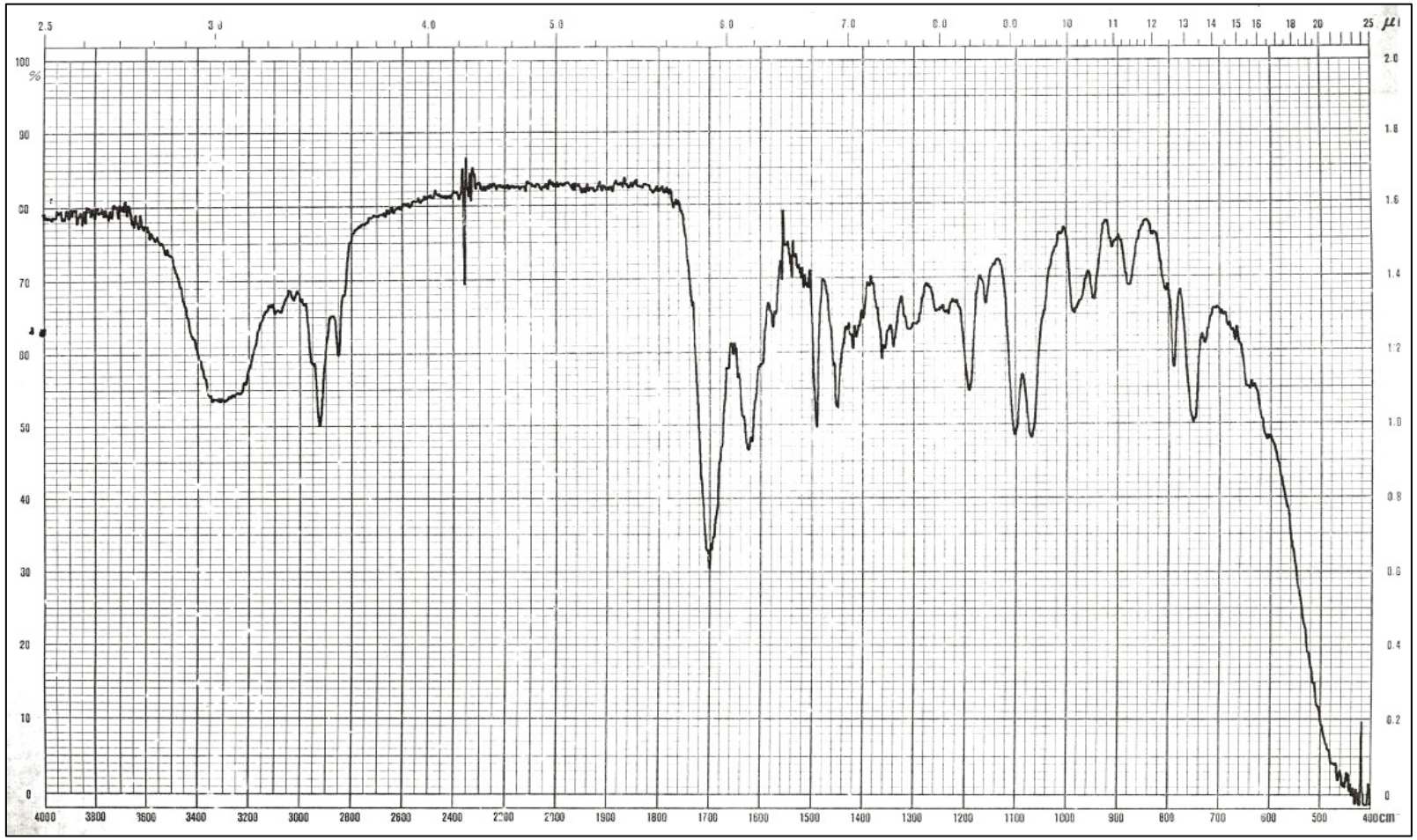
HMQC spectrum of compound **G9** (500 MHz, $CDCl_3$)



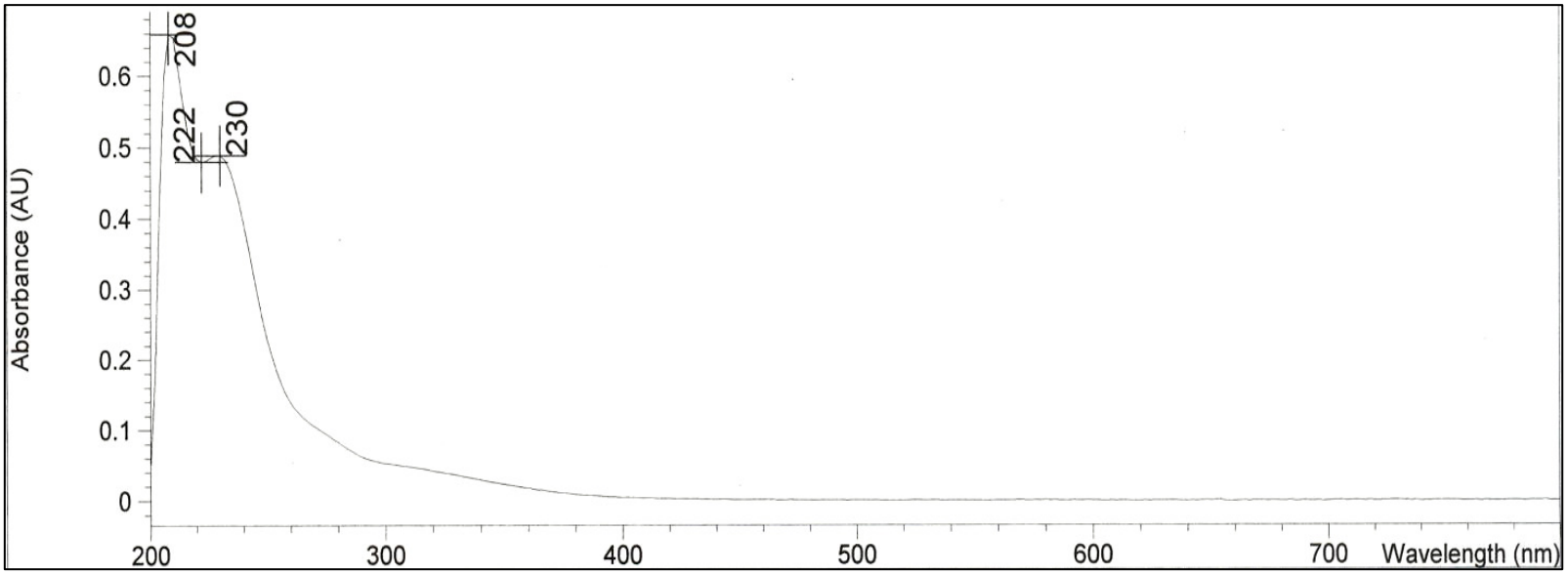
HMBC spectrum of compound **G9** (500 MHz, $CDCl_3$)



EIMS spectrum of compound **G9**



IR spectrum of compound **G9**



UV spectrum of compound **G9**

VITAE

Name Mr. Yutthapong Sangnoi

Student ID 4683004

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Aquatic Sciences)	Prince of Songkla University	2003

Scholarships and Award during Enrolment

- Academic Excellence Enhancement Program in Agro-Industry, Prince of Songkla University, 2004-2006

- Graduate School, Prince of Songkla University, 2003-2005

- First place-poster presentation (Industrial biotechnology and bioprocess engineering) The 18th Annual Meeting of the Thai Society for Biotechnology. Biotechnology: Benefits and Bioethics. November 2-3, 2006

List of Publication and Proceeding

Publication

Srisukchayakul, P., Suwannachart, C., Sangnoi, Y., Kanjana-Opas, A., Hosoya, S., Yokota, A. and Arunpairojana, V. 2007. *Rapidithrix thailandica* gen. nov., sp. nov., a marine gliding bacteria isolated from Andaman sea, the southern coastline of Thailand. Int. J. Syst. Evol. Microbiol. 57: 2275-2279.

Proceedings

Sangnoi, Y., Arunpairojana, V., Plubrukarn, A. and Kanjana-Opas, A. Exploitation of Marine Gliding Bacteria as the Source of Cytotoxic Compounds. The 18th Annual Meeting of the Thai Society for Biotechnology. Biotechnology: Benefits and Bioethics. Montien Hotel, Bangkok, Thailand. November 2-3, 2006. (Poster presentation)

Sangnoi, Y., Plubrukarn, A., Arunpairojana, V. and Kanjana-Opas, A. Secondary Metabolites from Novel Marine Gliding Bacteria. The 11th International Conference on Culture Collections. Goslar, Germany. October 7-11, 2007. (Poster presentation)

Sangnoi, Y., Srisukchayakul, P., Suwanachart, C., Arunpairojana, V., Plubrukarn, A. and Kanjana-Opas, A. Cytotoxic Compounds from Marine Gliding Bacteria in Thailand. The 1st Seminar of Bioactive Natural Products from Marine Organisms and Endophytic Fungi and The JSPS 2nd Medicinal Chemistry Seminar of Asia/Africa Science Platform Program. Phuket, Thailand. October 25-28, 2007. (Oral poster presentation)