สำนักงานกองทุนสนับสนุนการวิจัย
The Thailand Research Fund

Final Report

Project Title: Study of the chemical components of toxin derived from *Hirsutella thompsonii* (Fisher) in Thailand

Investigator: Saowanit Maimala
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This work was supported by Thailand Research Fund (TRF) under the Royal Golden Jubilee project

ACKNOWLEDGEMENTS

I wish to express my sincere appreciation to my thesis advisor; Professor Dr. Angsumarn Chandrapatya who extends valuable suggestions and criticism throughout the course of the project, encouragement, kindness and correcting the manuscript throughout the course of my study.

I am indebted to Dr.Prasat Kittakoop and all my friends in his lab (Bioresources Research Unit, BRU), my grateful to Miss Sukitaya Veeranondha (Animal Cell Biotechnology Laboratory), both of the National Center for Genetic Engineering and Biotechnology (BIOTEC), National Science and Technology Development Agency (NSTDA) who gave valuable suggestions and technical support about chemical component analysis and the cytotoxicity test during my study.

Finally, my special thank goes to the Thailand Research Fund (TRF) who provided financial support to me through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0114/2542) under project code: BGJ4580003.

Saowanit Maimala

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Project Period: 1 year (1 May 2002 – 1May 2003)

Abstract

Hirsutella thompsonii # 966 cultured in malt extract broth for 1 month without air flow was used to analyze chemical components by extraction with EtOAC. The components were separated with a Sephadex LH-20 column and analyzed on a Bruker DRX 400 NMR spectrometer. No new compounds appeared in the purification process, which showed only a group of ordinary components: peptide, glycerol derivative, ergosterol and diketopiperazines A and B, so, the extraction process was terminated. The crude broth (freeze dried sample) and the crude extract from broth (after evaporation) were used to investigate the cytotoxicity against insect and mammalian cell lines by dissolving in dimethyl sulfoxide (DMSO). Two insect cell lines, Sf9 (pupal ovarian tissue of *Spodoptera frugiperda* Smith = ECACC No. 89070101) and Clone C6/36 (larvae tissue of *Aedes albopictus* Skuse = ECACC No. 89051705) plus one mammalian cell line, clone BHK (21) clone 13 (Hamster Syrian kidney = ECACC No. 85011433) were used in this study. A bioassay revealed that the crude broth sample was weakly toxic to all cell lines in the tested range of 7.8-1,000 µg/ml while the crude extract was non-toxic to all cell lines tested in the range of 0.78-100µg/ml.

Key word: *Hirsutella*, toxin, cytotoxicity, bioassay

Introduction

Hirsutella thompsonii (Fisher) is an important naturally occurring fungal pathogen of eriophyoid mites, especially citrus rust mite (*Phyllocoptruta oleivora* Ashmead) which inhabits numerous host plants in subtropical and tropical regions (Jeppson *et al.*, 1975; Keifer *et al.*, 1982; Boczek *et al.*, 1989). *H. thompsonii* is a parasitic fungus belonging to the Phylum Deuteromycota (Fungi Imperfecti), Order Moniliales (McCoy and Kanavel, 1969) and first found in citrus orchards in Florida, USA (Speare and Yothers, 1924). In 1969, McCoy and Kanavel isolated this fungus on an artificial medium and confirmed its pathogenicity to mites. The infection started from conidia attached to the mite's cuticle and penetrated into its body. Hyphae ramified within the haemocoel before moving through the legs, mouth and anal opening. Conidia required a minimum of 4 h to penetrate the cuticle and completed its life cycle in the body of the mites around 72 h. Sporulation occurred within 9-24 h after host death. The fungus required at least 98% relative humidity (RH) and an optimal temperature of 25-30 °C for growth and development (McCoy, 1979; Gerson *et al.*, 1979; Kenneth *et al.*, 1979).

In 1992, Mazet identified two distinct protein toxins, hirsutellin A (HtA) and hirsutellin B (HtB) from *H. thompsonii* var. *thompsonii* (strain HTF-87) in a shake flask. Later, HtA was found to be highly toxic to *Galleria mellonella* (Linnaeus) larvae, *Drosophila melanogaster* (Meigen) adults, a cell line of *Bombyx mori* (Linnaeus) and *Spodoptera frugiperda* (Smith) larvae (Vey *et al.*,1993; Liu *et al.*, 1996). Additionally, Omoto and McCoy (1998) reported that HtA increased mortality of *P. oleivora* and also decreased its fecundity.

In Thailand, *H. thompsonii* var. *synnematosa* was first recorded on the citrus rust mite (*P. oleivora*) infesting citrus plants at Klong 7, Amphor Lum Luk Ka, Pathum Thani province (Chandrapatya, 1987). Preliminary investigation revealed that this fungus could be mass-produced successfully in both solid and liquid cultures. Maimala *et al.* (2002) studied 125 isolates of *H. thompsonii* collected from Thailand compared with several isolates of *H. thompsonii* kept in the collection of Insect pathology laboratory, University of Florida, USA. The HtA gene in each isolate was detected using the HtA gene of *H. thompsonii* strain JAB04, GenBank Accession Number

U86836 (Boucias *et al.*, 1998). Subsequently, crude broth of each isolate was assayed against *G. mellonella* larvae. The results showed that 100 out of 162 isolates had the HtA gene and more than half of the broth filtrates exhibited >50% mortality when assayed against the larvae. Unfortunately, the presence of the HtA gene in some isolates was not associated with enhanced insecticidal activity. In addition, the results from both isolate groups (with or without an amplifiable HtA gene) induced a relatively similar mortality rate (65%) in the larvae. The conclusion suggested that there was no direct correlation between the presence of the PCR-amplified HtA product and the insecticidal activity against *G. mellonella* larvae. Hence, this fungus must be secreting other toxins that have not yet been characterized.

The objective of this study was to analyze chemical components of *Hirsutella* toxins and investigate their activity against insect and mammalian cell lines.

Materials and Methods

H. thompsonii # 966 was cultured on Malt extract agar (MEA) for 7 days before 5 ml of sterile distilled water and 0.5% Tween was added to each petri-dish. Each fungal colony was scrapped off with a sterile spatula and transferred to 250 ml Erlenmeyer flasks containing 100 ml of Malt extract broth (MEB). The flasks were incubated on a rotary shaker at 27°C, 180 rpm for 4 consecutive days. The fungal biomass in each flask was blended in an electrical blender and 2 ml transferred to a new MEB flask and continued to culture on the shaker under the same conditions for 4 days. This biomass was used as inoculum throughout the experiments.

Toxin extraction and isolation

Inoculum of *H. thompsonii* # 966 (20 ml) was added to a 4 L Erlenmayer flask containing 2 L of MEB. *Hirsutella* fungus was cultured without air flow and incubated at room temperature for 1 month (3 flasks/treatment).

When the fungal culturing was terminated, fresh biomass was separated from the broth by filtration through Whatman #1 filter paper. Broth was put through extraction 3 times in a separating funnel using ethyl acetate (EtOAc) in an equal ratio with crude broth. The aqeuous solution on the top layer (ethyl acetate layer) was collected and evaporated yielding concentrated crude extract. This crude extract was divided into 2 parts. The first part was loaded into a Sephadex LH-20 (eluted with MeOH) to separate chemical components and then a Bruker DRX 400 NMR spectrometer operating at 400 MHz for proton and 100 MHz for carbon was used to analyse the chemicals present in the crude extract (Vongvanich *et al.*, 2002). The second part was used to determine the toxicity of the crude extract against insect and mammalian cell lines.

Fresh biomass was extracted by soaking in methanol (MeOH) for 2 days before filtering and subsequently soaking in dichloromethane (CH_2Cl_2) for a further 2 days. Both methanol and dichloromethane extractants were evaporated separately to concentrate the soluble materials. Concentrated crude extracts collected from MeOH and CH_2Cl_2 were then extracted together 3 times with EtOAc. This combination was divided into two layers, the top layer was EtOAc (non-polar part) and the bottom layer was H_2O (polar part). Both layers were evaporated before loading into a Sephadex LH-20 and then chemical components were analyzed in the Bruker DRX 400 NMR spectrometer. Interesting fractions were reloaded in the column and NMR run until a chemical compound was obtained (Chart 1-4).

Bioassay of crude extract against cell lines

H. thompsonii was cultured in 250 ml Erlenmeyer flasks containing 100 ml of Malt extract broth (MEB) and incubated on a rotary shaker at 27°C, 180 rpm for 4 days. The crude broth was then separated from the mycelia before being evaporated and used in the bioassay test.

Both crude broth and crude extract from the toxin extraction process were dissolved in dimethyl sulfoxide (DMSO) to give a stock concentration of 100 mg/ml.

This solution was further diluted (1:2) in the respective growth medium of each cell line over the range of 1mg to 7.8 µg/ml.

Cell culture

The cytotoxicity of *Hirsutella* fungus was assessed against two insect cell lines, Sf9 (pupal ovarian tissue of *Spodoptera frugiperda* Smith = ECACC No. 89070101) and Clone C6/36 (larvae tissue of *Aedes albopictus* Skuse = ECACC No. 89051705) plus one mammalian cell line, clone BHK (21) clone 13 (Hamster Syrian kidney = ECACC No. 85011433).

The cell line Sf9 was grown in TC100 insect medium supplemented with 2mM L-glucosamine, 100U/ml Penicillin/Streptomycin and 10% fetal bovine serum. C6/36 was grown in Minimum Essential Medium (MEM) supplemented with 2mM L-glutamine, 1% MEM-NEAA, 100 U/ml Penicillin/Streptomycin and 10% fetal bovine serum. These insect cells were incubated at 28°C. The mammalian cell line (BHK (21) clone 13) was grown in Glasgow MEM (GMEM) supplemented with 2mM L-glutamine, 100U/ml Penicillin/Streptomycin and 5% fetal bovine serum, and the cells were incubated at 37°C in a fully humidified, 5% CO_2: air atmosphere.

Cytotoxicity test

Three cell lines (Sf9, C6/36 and BHK (21) clone 13) were seeded separately in three 96-well plates at, 10,000, 20,000 and 1,000 cells/well/plate, and incubated for 48 h. The 1:2 serial diluted crude extracts were added to the cells and incubated for 24 h. Crude extracts were then removed from the cell cultures and the cells reincubated for another 24 h in fresh medium and then tested with MTT assay which is an indirect measurement of cell viability. This assay is a tetrazolium-dye based colorimetric microtitration assay where metabolism-competent cells are able to metabolize the tetrazolium (yellow) to formazan (blue). This color change is measured spectrophotometrically with a plate reader. It is assumed that cells that are metabolically deficient will not survive (Skehan *et al.*, 1990).

A dose-response curve was derived from 8 concentrations in the test range of 1000-7.8 µg/ml using 4 wells/concentration. Within each experiment 3-dose response curves were obtained. Toxicity was expressed as the concentration of the sample required to kill 50% (LD_{50}) of the cells in comparison to the controls.

Results and Discussion

Toxin extraction and isolation

The analysis of biomass extracts revealed that the culture grown in MEB for 1 month without air flow contained some interesting chemical components. This result indicated that the fungus cultured in this substrate for a long period produced or released some chemical components.

The crude broth (6 L) extracted with EtOAc 3 times yielded a total of 0.26 g concentrated crude extract. This crude extract was then dissolved with MeOH and the components separated by loading through the Sephadex LH-20 (eluted with MeOH). Fractions 1-6 were examined with the Bruker DRX 400 NMR spectrometer, and the forth fraction was subsequently rechromatographed through Sephadex LH-20, yielding a pure peptide of 0.017 g (Chart 2). The ^{1}H and ^{13}C NMR spectra ($CDCl_3$) of this isolated peptide are shown in Figures 1 and 2.

Sequential extracts from mycelia with MeOH and CH_2Cl_2 produced a combined total crude extract of 0.26 g. This combined extract was then partitioned with H_2O/EtOAc. The crude extracts from the polar part (H_2O) and non-polar part (EtOAc) were 0.185 g and 1.83 g respectively. When the crude extract from the non-polar part was repeatedly purified with gel filtration on Sephadex LH-20 a glycerol derivative (0.009 g) and ergosterol (0.017g) were obtained (Chart 3). The ^{1}H NMR spectrum of the glycerol derivative (DMSO-d6) is shown in Figure 3, while that of ergosterol ($CDCl_3$) is shown in Figure 4. The H_2O soluble (polar part) was also purified by Sephadex LH-20 column chromatography to yield diketopiperazines A and B, of 0.105 g and 0.071 g respectively (Chart 4). The ^{1}H and ^{13}C NMR spectra (D_2O) of diketopiperazine A are

shown in Figures 5 and 6, while the ^{1}H NMR spectrum (D_2O) of diketopiperazine B is illustrated in Figure 7.

A toxin protein called hirsutellin A (HtA) has been separated from *H. thompsonii* (strain HTF 87) by using ammonium sulfate precipitation, ion exchange chromatography and gel filtration on Bio-gel P-10. The result indicated that hirsutellin A (HtA) comprised only one copy of a peptide chain. Hirsutellin A comprised of 13 amino acids: lysine, aspartic acid + asparagine, alanine, glycine, glutamic acid + glutamine, valine, arginine, proline, threonine, isoleucine, phenylalanine, leucine and tyrosine (Mazet and Vey, 1995). Liu *et al.* (1995) also extracted and characterized hirsutellin A from *Hirsutella thompsonii* var. *thompsonii* (strain JAB-04) isolated from citrus rust mite by using a combination of ion-exchange, gel-permeation and immunoaffinity chromatography. This result showed a composition of amino acids which is quite similar to that of Mazet and Vey (1995). In 1998, Boucias *et al.* reported that an extracellular insecticidal protein (HtA) produced by *H. thompsonii* contained 14 lysines, 7 arginines and 3 histidines. The sequence of HtA is unique and does not produce the secondary or tertiary structures characteristic of other fungal RIPs. In addition, Vongvanich *et al.* (2002) cultured *Hirsutella kobayashi* (BCC 1660) isolated from a cricket, in potato dextrose broth (PDB) (5 L) at 25°C for 21 days. Crude broth was extracted with EtOAc and mycelia with MeOH and CH_2Cl_2. The crude extract was purified by Sephadex LH-20 (eluted with MeOH) and silica gel column chromatography to yield a new cyclohexadepsipeptide, named hirsutellide A. Hirsutellide A exhibited antimycobacterial and antimalarial activities but was inactive against the Vero cell line.

In this study, structure elucidation of the isolated compounds was not completed, showing only a group of ordinary components. However, further study on the identification of chemical structures of these isolated compounds would be of great interest.

Cytotoxicity test

The bioassay of fungal crude broth (freeze-dried sample) with insect and mammalian cell lines revealed that the crude broth was weakly toxic to all cell lines in

the tested range of 7.8-1,000 µg/ml. The ID50 of the tested sample with Sf9, C6/36 and BHK (21) clone 13 were >1,000, 804±85 and 914±14 µg/ml, respectively. The C6/36 and BHK (21) clone 13 cell lines treated with 1,000 µg/ml of crude broth survived only 46.42% and 48.08%, respectively. Cell lines in other treatments survived more than 50% indicating no cytotoxicity (Table 1).

Fungal crude extract (after evaporation) from broth was non-toxic to all tested cell lines in the range of 0.78-100 µg/ml. Cell lines in all treatments survived more than 70%. Interestingly, the numbers of viable BHK(21) cells were considerably higher than the controls (Table 2). These results indicate that BHK(21) cells were more sensitive than SF9. The crude extract used in this experiment probably stimulated cell activity, and therefore, fungal crude extract from broth is safe and non-toxic to mammalian cells.

Effectiveness of *Hirsutella* depends on the fungal variety and cultured conditions, for example, hirsutellin A (HtA) extracted from *H. thompsonii* var. *thompsonii* (strain HTF-87) cultured in Czapek-Dox broth plus yeast extract is highly toxic to *Galleria mellonella* (Linnaeus) larvae and *Aedes aegypti* (Linnaeus). HtA extracted from *H. thompsonii* var. *thompsonii* (strain JAB 04) cultured in PDY broth media is also toxic to *G. mellonella* (Linnaeus) larvae. Moreover, HtA extracted from *H. thompsonii* var. *thompsonii* (strain CBS 556.77D.) cultured in Czapek-Dox broth plus yeast extract showed high toxicity to *G. mellonella* (Linnaeus) larvae, *Drosophila melanogaster* (Meigen) adults and cell line of *Bombyx mori* (Linnaeus) (Vey *et al.*, 1993; Liu *et al.*, 1995; Mazet and Vey, 1995). In contrast, when Vongvanich *et al.* (2002) extracted hirsutellide A from *H. kobayashi* (BCC 1660) cultured in potato dextrose broth (PDB), hirsutellide A exhibited antimycobacterial and antimalarial activities with a MIC (minimum inhibitory concentration) of 6-12 µg/ml. but showed no cytotoxic effect toward the Vero cell line at 50 µg/ml.

Conclusion

In this study, structure elucidation of the isolated compounds was not completed, showing only a group of ordinary components. In addition, no new compounds appeared in the purification process. Unfortunately, the quantity of crude extract used in this experiment was not enough to induce cytotoxicity. Bioassay tests of the crude extract showed no toxicity to any cell lines. When the crude broth (freeze-dried sample) was tested against all cell lines at the concentration of 7.8-1,000 μg/ml, only crude broth at the concentration of 1,000 μg/ml was found to be weakly toxic to cells line of C6/36 and BHK(21). Further study on the identification of chemical structures of these isolated compounds is still of great interest.

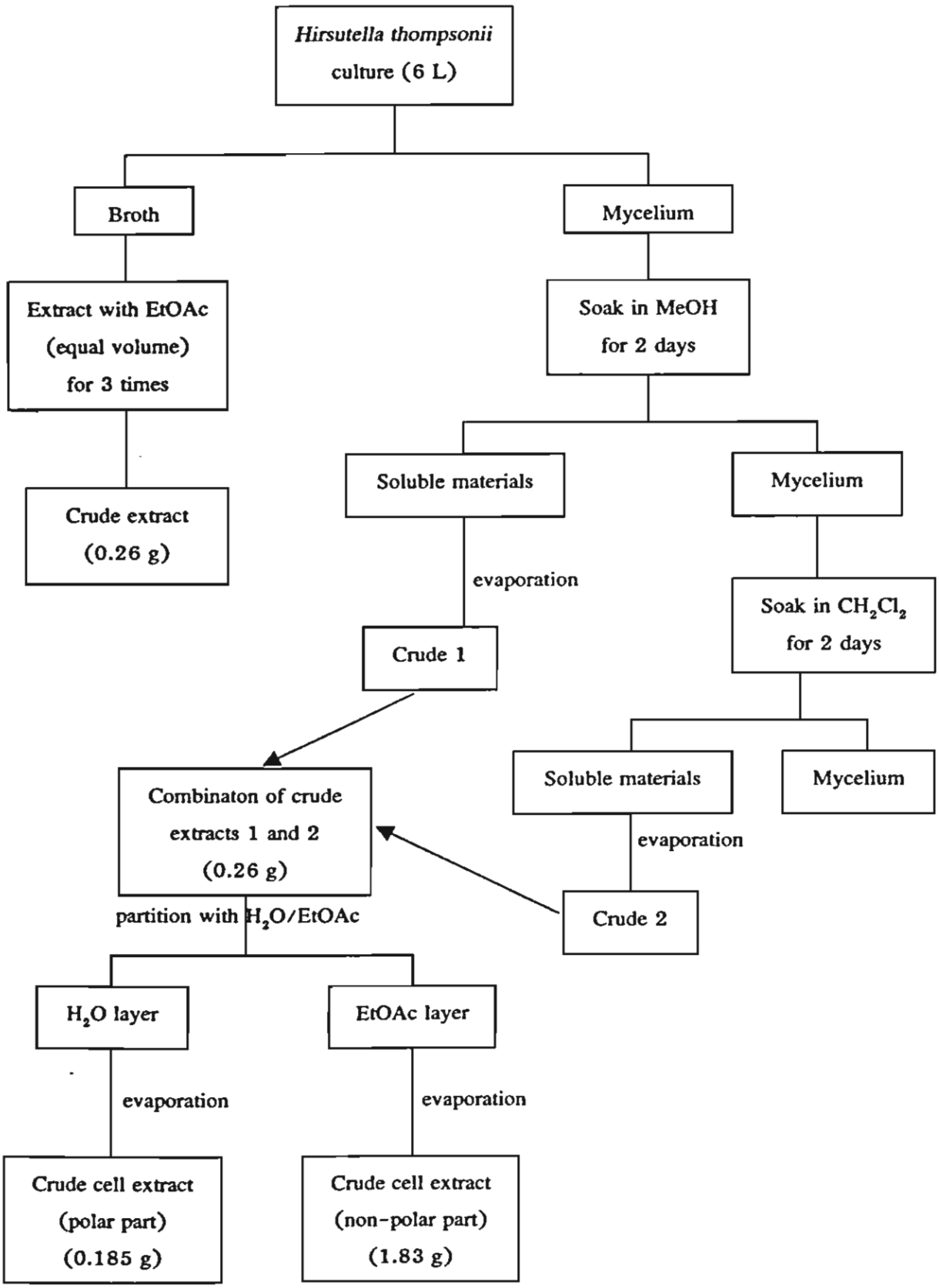

Chart 1 Fungal extraction and isolation

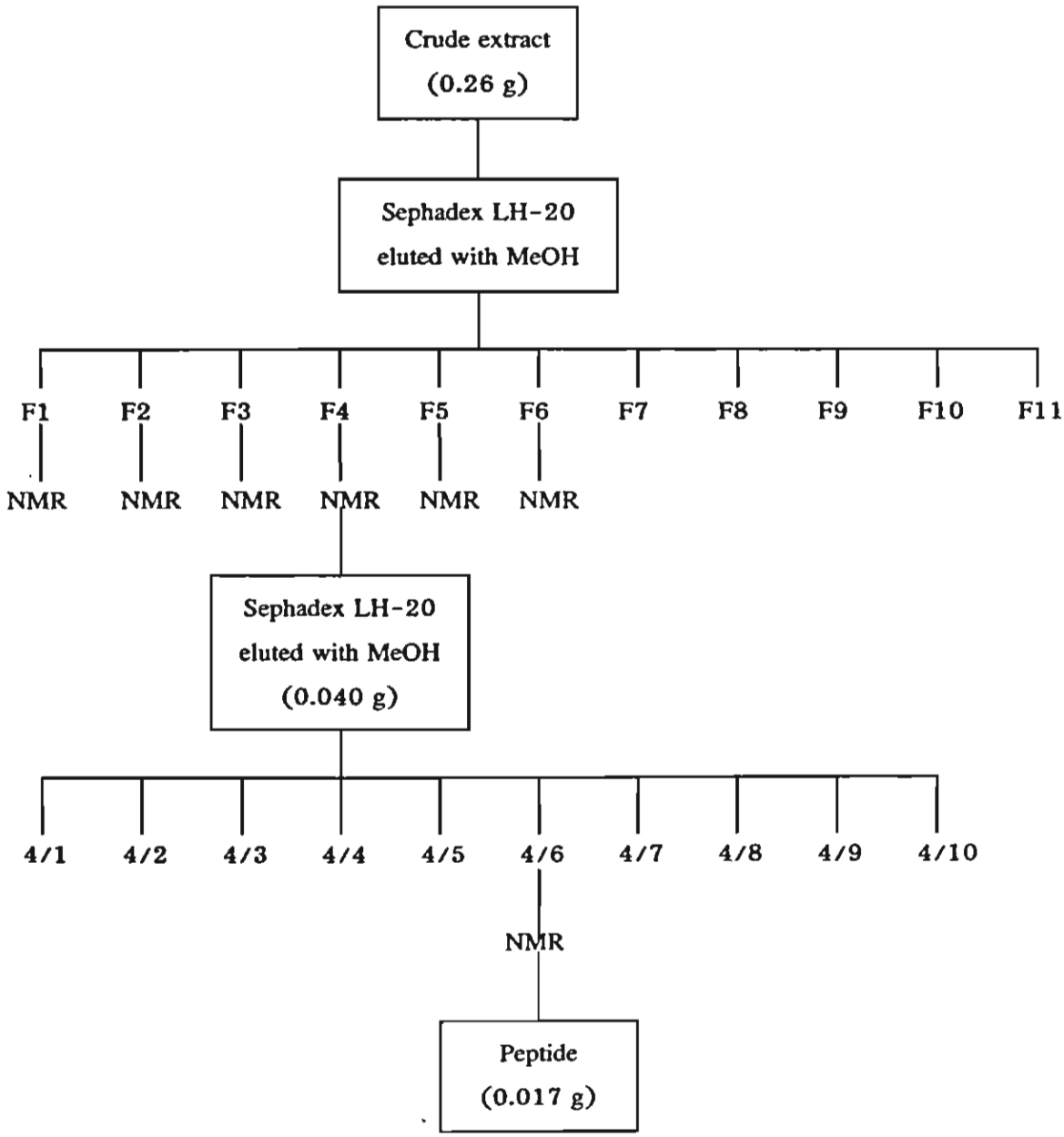

Chart 2 Crude extract from broth

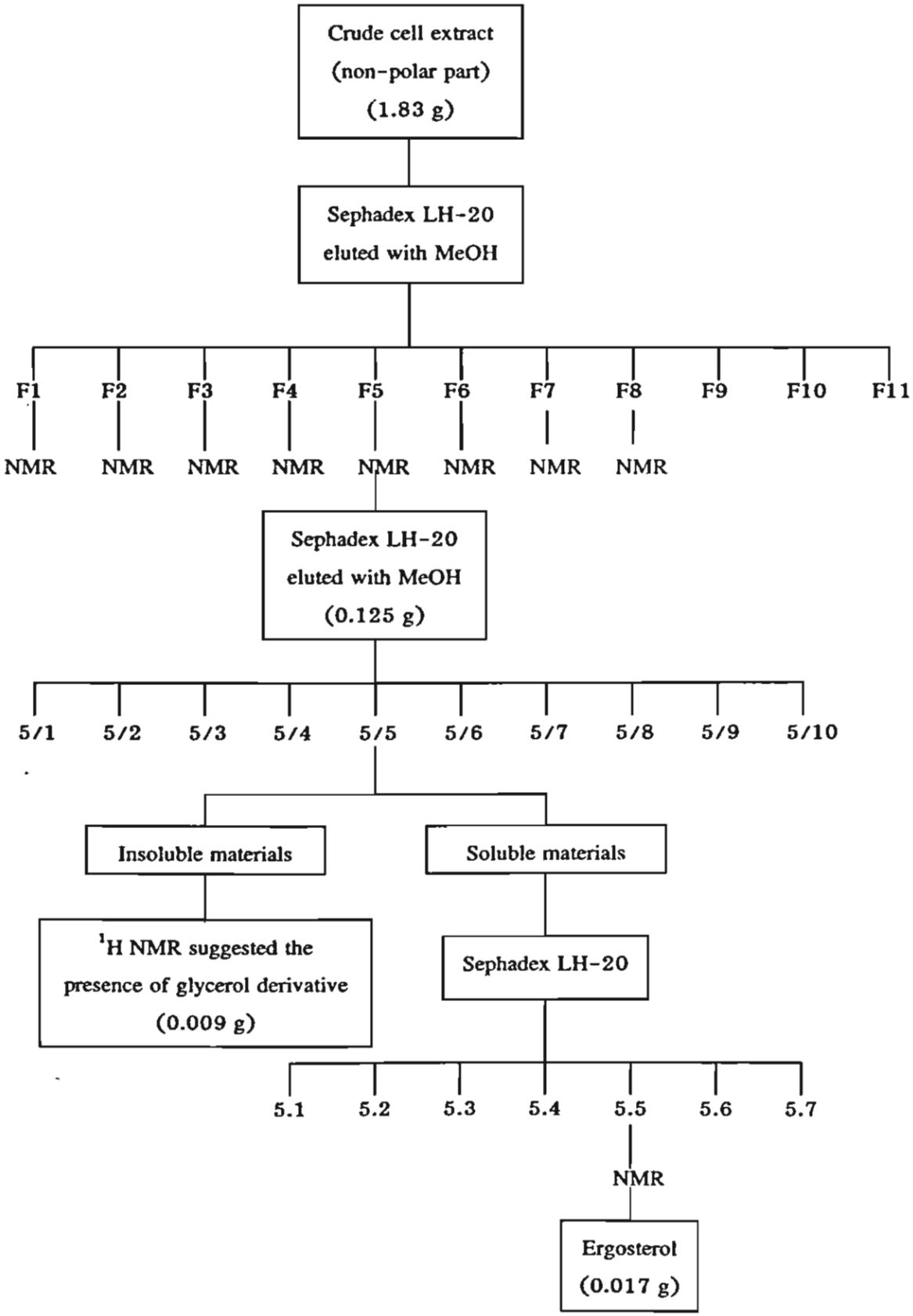

Chart 3 Isolation of crude cell extract (non-polar part)

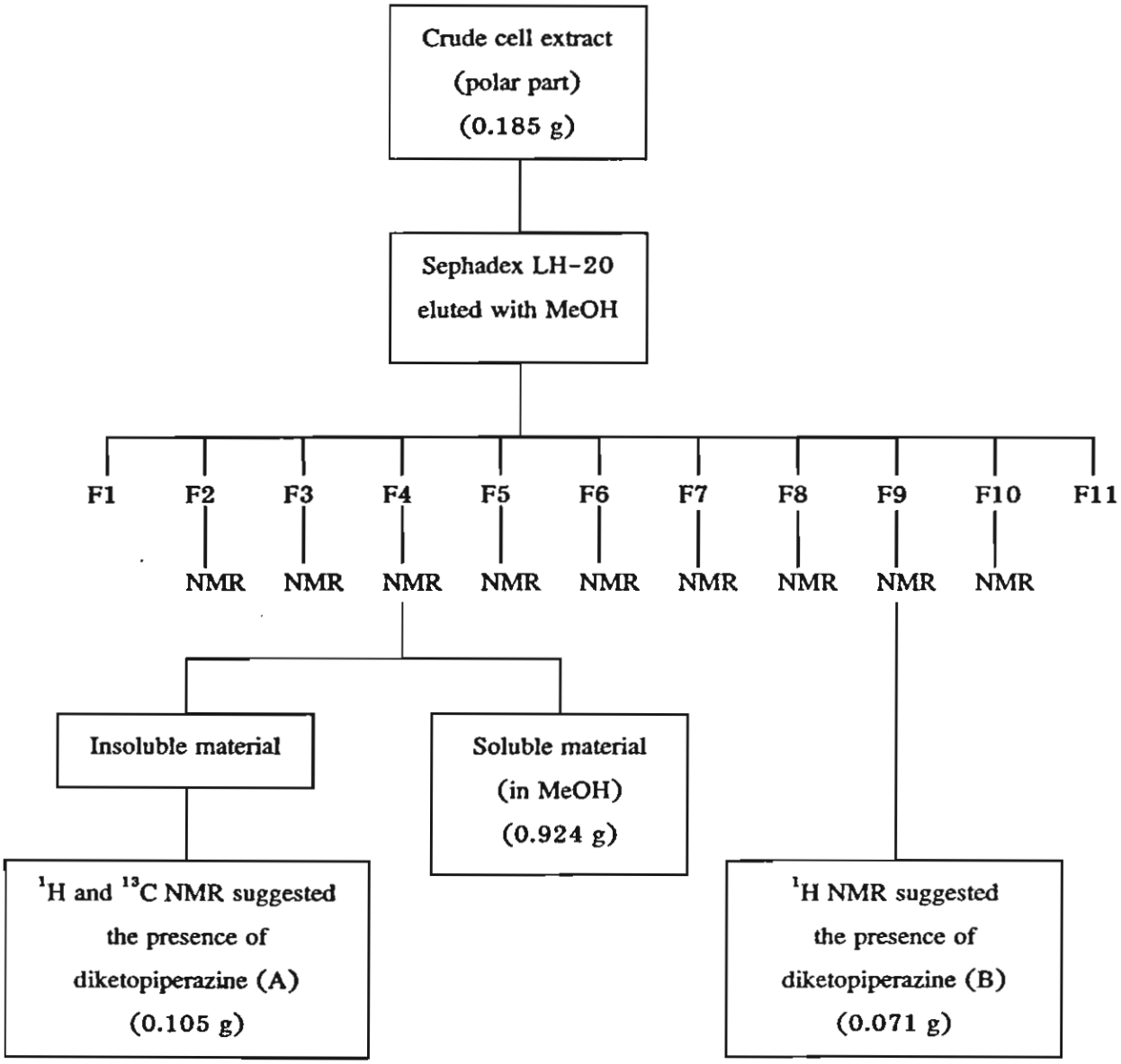

Chart 4 Isolation of crude cell extract (polar part)

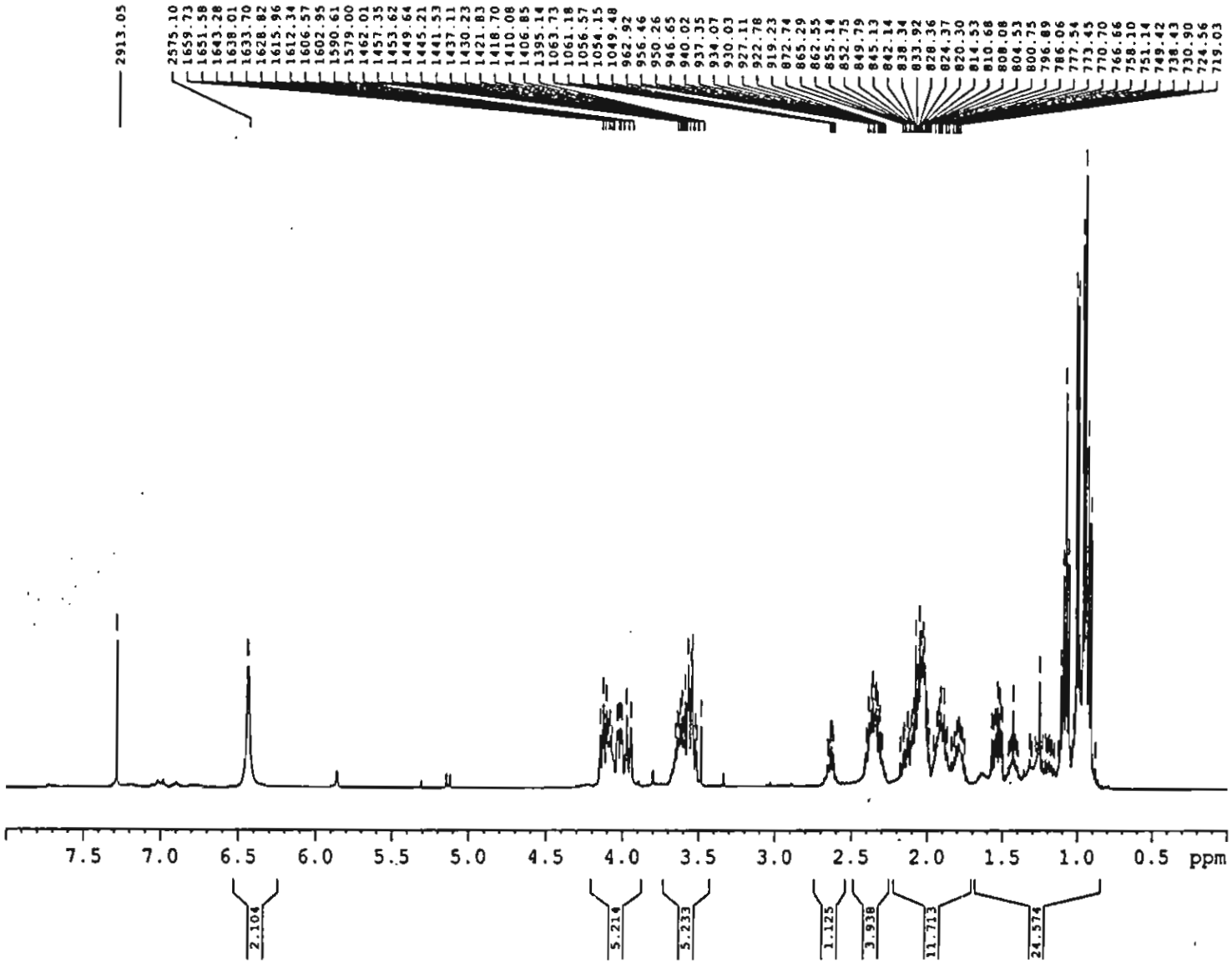

Figure 1 The ^{1}H NMR spectrum ($CDCl_3$) of the isolated peptide

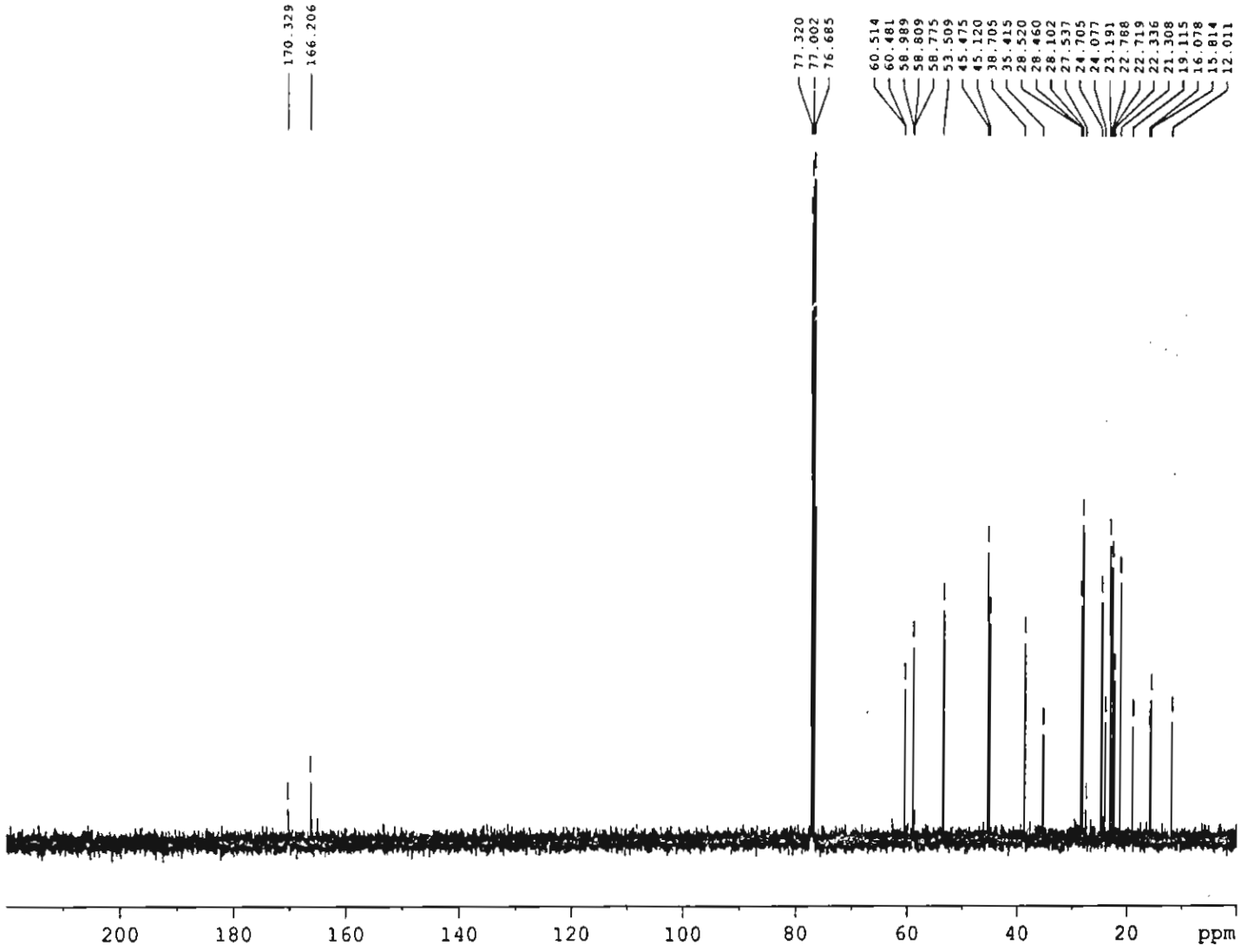

Figure 2 ^{13}C NMR spectrum ($CDCl_3$) of the isolated peptide

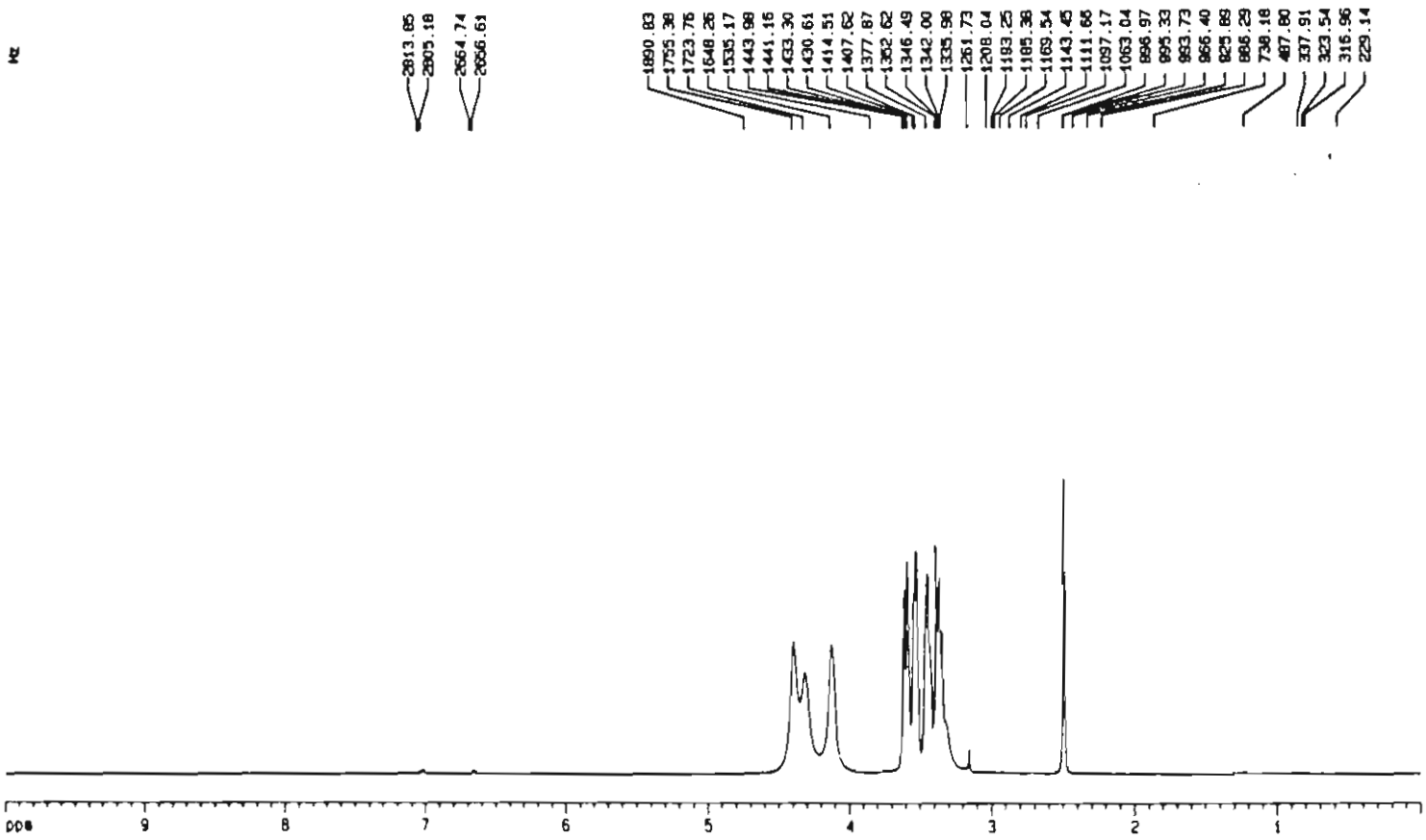

Figure 3 ^{1}H NMR spectrum of the glycerol derivative (DMSO-d6)

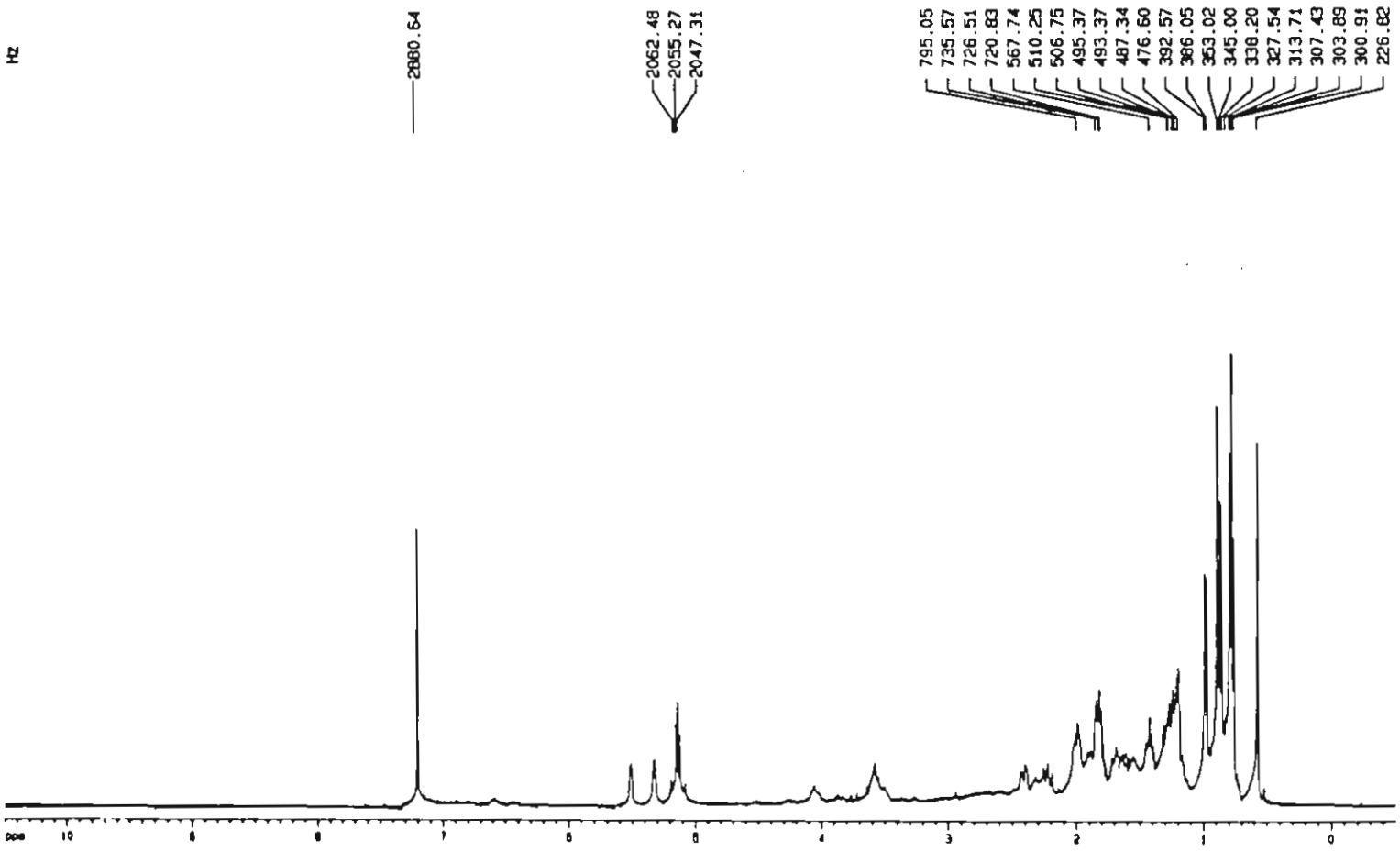

Figure 4 ^{1}H NMR spectrum of ergosterol ($CDCl_3$)

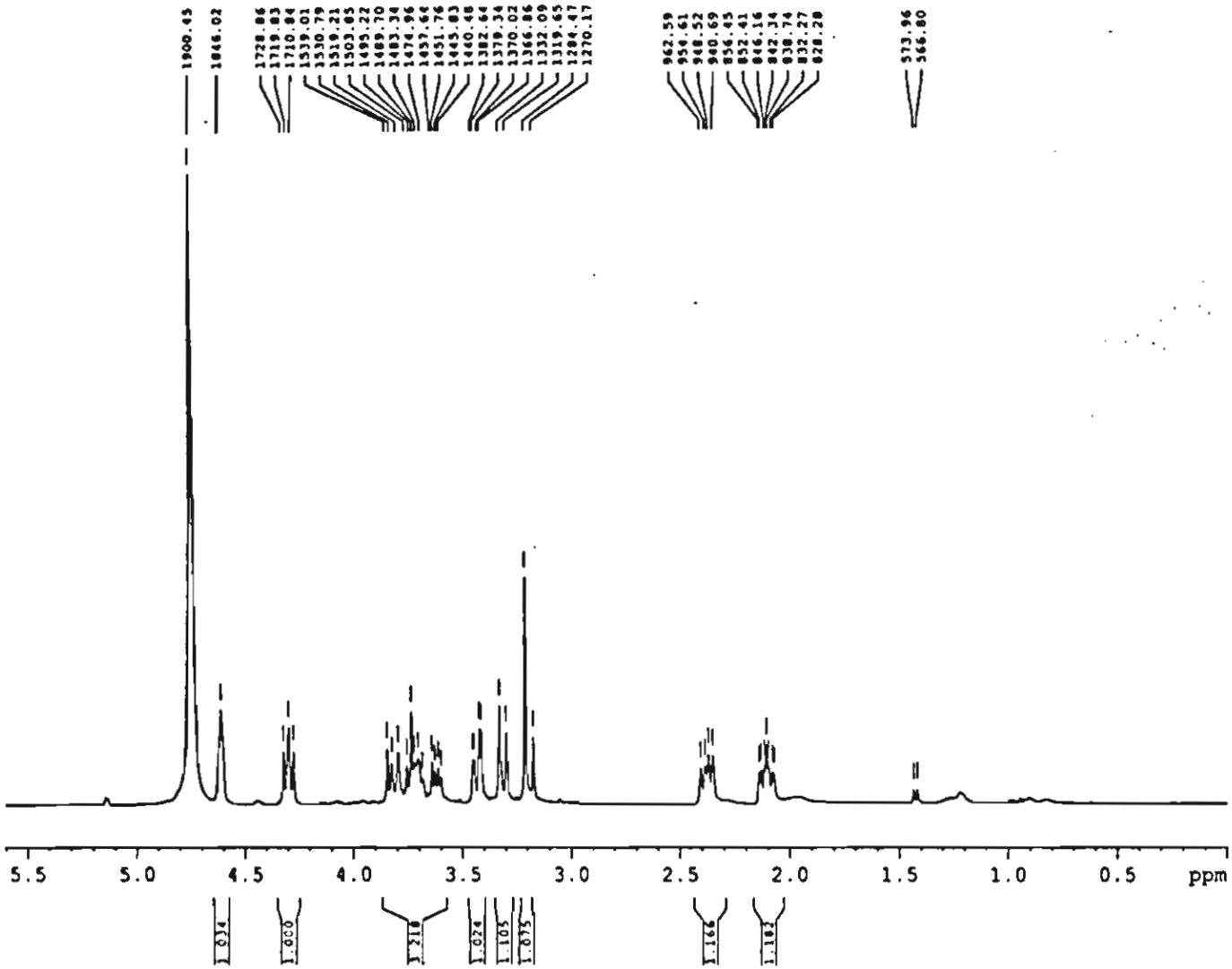

Figure 5 ^{1}H NMR spectrum (D_2O) of diketopiperazine A

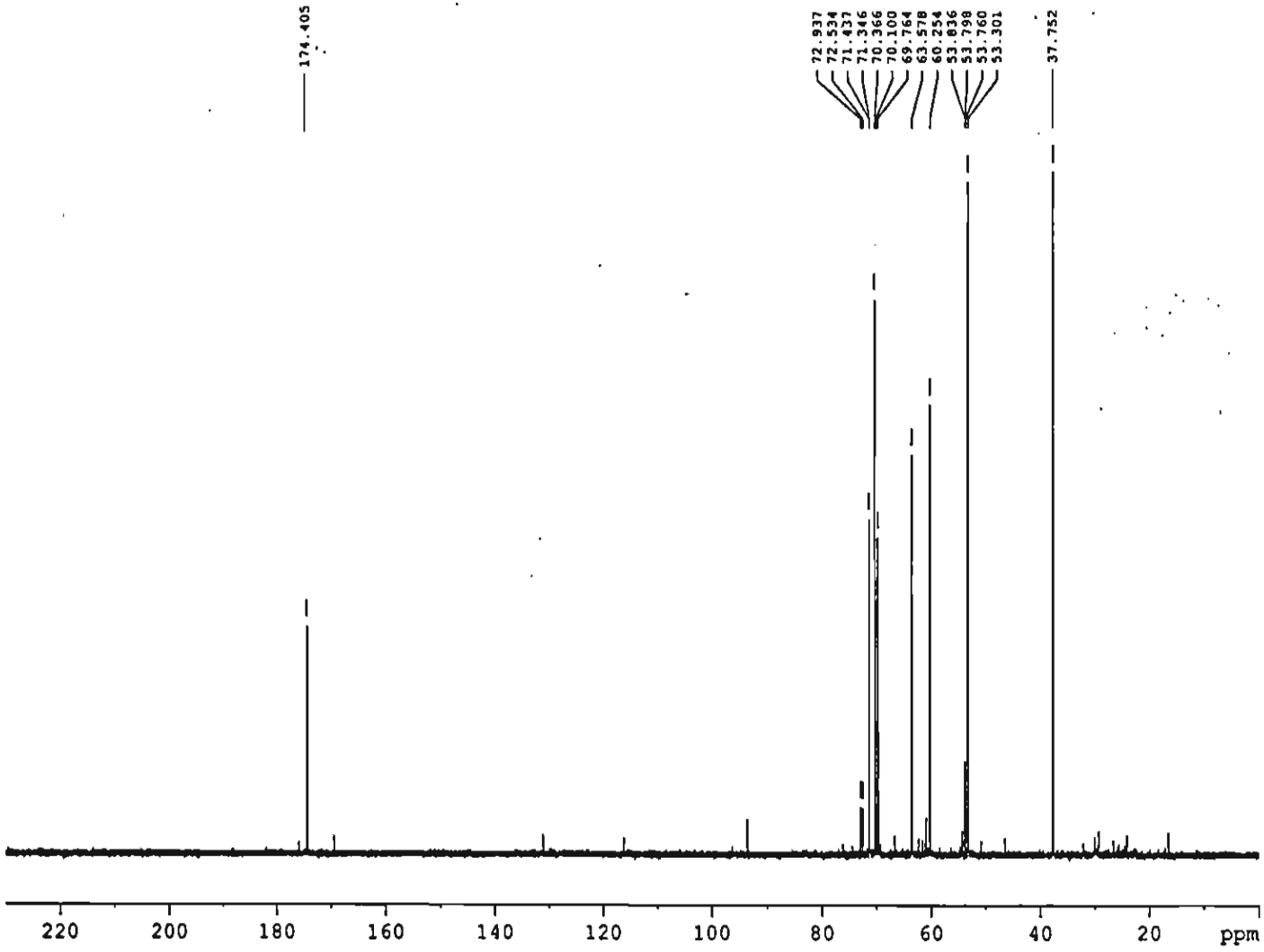

Figure 6 ^{13}C NMR spectrum (D_2O) of diketopiperazine A

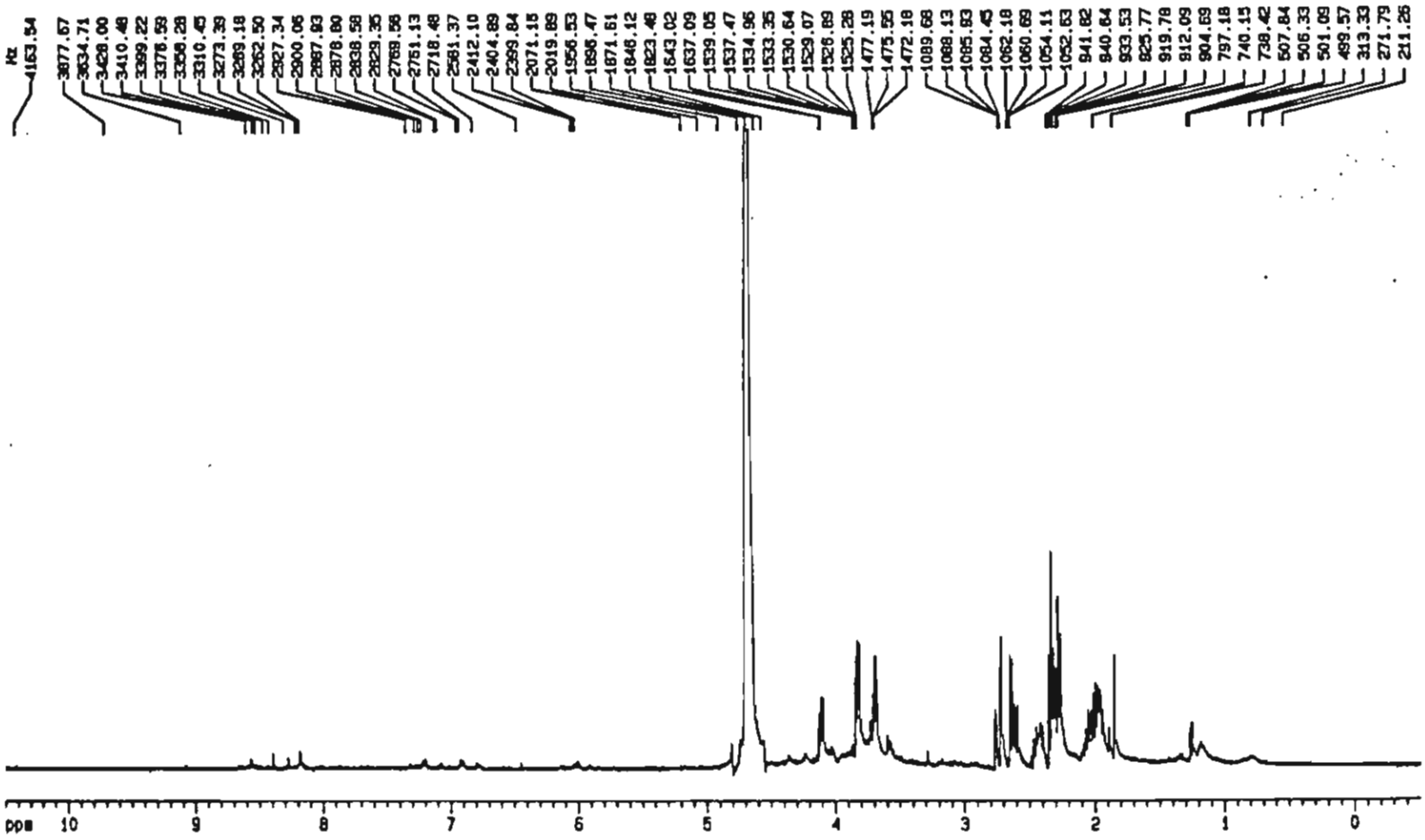

Figure 7 ^{1}H NMR spectrum (D_2O) of diketopiperazine B

Table 1 Optical density (OD) and survival percentage of different cell lines after treatment with crude broth of *H. thompsonii* #966 for 24 hours.

Standard (μg)	SF9		C6/36		BHK(21)	
	Mean OD	% survival	Mean OD	% survival	Mean OD	% survival
1000	0.65	74.71	0.52	46.42	0.25	48.08
500	0.75	86.21	0.63	56.25	0.31	59.62
250	0.83	95.40	0.69	61.61	0.36	69.23
125	0.81	93.10	0.76	67.86	0.40	76.92
62.5	0.85	97.70	0.83	74.11	0.42	80.77
31.25	0.89	102.30	0.97	86.61	0.46	88.46
15.63	0.88	101.15	1.15	102.68	0.49	94.23
7.8	0.93	106.90	1.19	106.25	0.50	96.15
Control	0.87	100	1.12	100	0.52	100

Table 2 Optical density (OD) and survival percentage of different cell lines after treatment with crude extract from broth of *H. thompsonii* #966 for 24 hours.

Standard (μg)	SF9		C6/36		BHK(21)	
	Mean OD	% survival	Mean OD	% survival	Mean OD	% survival
100	0.38	79.17	0.52	100	0.39	121.88
50	0.40	83.33	0.54	103.85	0.44	137.5
25	0.44	91.67	0.58	111.54	0.47	146.88
12.5	0.44	91.67	0.61	117.31	0.45	140.63
6.25	0.45	93.75	0.54	103.85	0.44	137.5
3.125	0.46	95.83	0.52	100	0.42	131.25
1.563	0.47	97.92	0.53	101.92	0.43	134.38
0.78	0.53	110	0.60	115.38	0.45	140.63
Control	0.48	100	0.52	100	0.32	100

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