Chemical constituents and cytotoxicity against breast cancer cells of South Australian marine sponge *Aplysilla rosea*

A thesis submitted in fulfilment of the requirement for the degree of Doctor of Philosophy

By

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Candidate's Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

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July 2016

Thesis Summary

Sponge [Porifera] is the richest source of marine natural products, representing over 30% of all marine natural products discovered by far. In Southern Australia Ocean, it is estimated there are over 800 marine sponge species; however, the discovery of marine natural products from these sponge species are very limited.

The aim of this study was, therefore, to screen untapped marine sponges from South Australian waters to discover sponge derived compounds with potential anti-breast cancer activity. Samples of thirty marine sponge species were collected and subjected to a standardised extraction protocol to produce crude extracts. These extracts were tested their cytotoxicity against three breast cancer cell lines (T47D, MCF7 and MDA-MB-231) and one human non-cancer breast cell line (184B5) using the Crystal Violet assay. 60% of the crude extracts showed cytotoxicity against three breast cancer cell lines with an IC50 value range between 5 mg/ml to 50 µg/ml. Crude extracts from three sponges (Tedania sp, Chondropsis cf. arenifera, and *Aplysilla rosea*) showed the highest toxic activity against three breast cancer cell lines with IC50 (48 h treatment) value of 4.05 to 6.62 µg/ml, 3.80 to 7.28 µ/ml and 8.31 to 12.57 µg/ml, respectively. The three extracts also had lower cytotoxicity against normal breast cell lines 184B5 with IC50 (48 h treatment) value of 14.59 µg/ml, 11.68 µg/ml and 21.96 µg/ml, respectively. Flow Cytometry was used to assess the ability of these three extracts in the apoptosis induction and cell cycle arrest of the three breast cancer cell lines. Extracts from Tedania sp and Chondropsis cf. arenifera showed the ability to induce cell apoptosis. The only extract from *Aplysilla rosea* showed activities in

both cell cycle arrest and apoptosis induction. Therefore, sponge *Aplysilla rosea* was chosen for further studies.

Using bioactivity-guided isolation, over 50 pure compounds have been obtained from sponge *Aplysilla rosea*, and 24 compounds were obtained in this thesis. Four novel aplyroseol-type diterpenes compounds (1, 2, 5, and 6) and seven know diterpenes (3, 4, 7-9, 14, and 16), eleven know steroids (10-13, 17-21, and 23-24) and two know fatty acids (15 and 22) were isolated from sponge *Aplysilla rosea*. The structures of these 24 compounds were elucidated by spectroscopic data (1D and 2D NMR, and HRESIMS). Compounds 1-7, 9, 13, 16, 19 and 24 showed cytotoxicity against three human breast cancer cell lines (T47D, MCF7 and MDA-MB-231), with IC50 value in the range of 3.3 to 16.2 µg/ml. In contrast, the IC50 value in the range of 5.6 to 18.6 µg/ml was indicated on normal breast cell line 184B5. Compound 7 exerted no cytotoxic effect on 184B5 cells after 48 h treatment, compared with IC50 values of 4.4 mg/ml, 3.5 mg/ml, and 3.3 mg/ml on T47D, MCF7 and MDA-MB-231, respectively. Compound 7 induced apoptosis induction and cell cycle arrest in T47D, MCF7 and MDA-MB-231 cell lines at G0/G1 phase.

In conclusion, this study has isolated and identified twenty-four compounds for sponge *Aplysilla rosea*, with several compounds demonstrated significant cytotoxicity against breast cancer cells. These results indicated a significant advance in the understanding of chemical constituent of sponge *Aplysilla rosea* for natural product discovery, with four novel compounds and eighteen known compounds never reported from *A. rosea*. Additionally, at least another twenty compounds could be

further identified, with potentially six new compounds. South Australian marine sponges, represented by *Aplysilla rosea* in this study is a rich source of marine natural product for anticancer drug discovery. Nearly 50% of these compounds identified in this study showed anti-breast cancer activity. Compound **7** indicated selective cytotoxicity against breast cancer cells over normal cells, which showed high potential for further study of the anti-cancer applications.

Keywords: Breast cancer; marine sponge; *Aplysilla rosea*; cytotoxicity; apoptosis; marine natural product

Acknowledgements and Dedication

First and foremost, I would like to pay the most important acknowledgement to my supervisors, Associate Prof Barbara Sanderson, Prof Wei Zhang and Prof Hou-Wen Lin, for their very great help in supervision of the project, my PhD candidature, and the laboratory.

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List of Abbreviations

%	Percentage
95% CI	95% confidence interval
ANOVA	Analysis of Variance
ATCC	American type culture collection
°C	Degree Celsius
CC	Column chromatography
$CDCl_3$	Deuterated chloroform
COSY	Correlation spectroscopy
d	double
DCM	Dichloromethane (CH_2Cl_2)
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
FBS	Foetal bovine serum
FDA	Food and drug administration
µg	Microgram
µl	Microliter
h	Hour
HCl	Hydrochloric acid
HSQC	Heteronuclear single quantum coherence
HMBC	Heteronuclear multiple bond correlation
ESIMS	Electrospray Ionization Mass Spectrometry
ER	Oestrogen receptor
HER2	Human epidermal growth factor receptor 2
HRESIMS	High resolution Electrospray Ionization Mass Spectrometry
HPLC	High performance liquid chromatography
IC_{50}	50% inhibitory concentration
J	Coupling constant (Hz)

m	multiplet
MeOH	Methanol
MBC	Metastatic breast cancer
ml	Millilitre
MeCN	Acetonitrile
MPLC	Medium pressure liquid chromatography
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
NMR	Nuclear Magnetic Resonance
NCI	National Cancer Institute
NOESY	Nuclear Overhauser effect spectroscopy
OD	Optical density
PBS	Phosphate-buffered saline
PI	Propidium iodide
PR	Progesterone receptor
q	quartet
ROESY	Rotational nuclear overhauser effect spectroscopy
RA	Regular approval
RCT	Randomized controlled trials
RPMI	Roswell Park Memorial Institute
RT	Room temperature
s	singlet
SEM	Standard error of the mean
SPSS	Statistical Package for the Social Sciences
t	triplet
t_R	Retention time
TLC	Thin-layer chromatography

Publications and presentations

Publications

- Shuang Peng, Barbara J.S. Sanderson, Hou-Wen Lin, Wei Zhang, Cytotoxicity extracts from South Australian marine sponges against human breast cancer cells (In submission)

- Shuang Peng, Barbara J.S. Sanderson, Wei Zhang, Hou-wen Lin, Aplyroseol 20-23, cytotoxic diterpenoids from South Australian marine sponge *Aplysilla resoa* (In preparation)

- Shuang Peng, Barbara J.S. Sanderson, Hou-wen Lin, Wei Zhang, Cytotoxic effect of Dendrillol-1 from marine sponge *Aplysilla rosea* on human breast cancer cell lines (In preparation)

- Guang-fei Wang, Rui-hua Ji, Meng-xue Cao, Wei-zhuo Tang, Hao-bing Yu, Bin-bin Gu, Li-jian Ding, Shuang Peng, Wei-hua Jiao and Fan Sun (2015). "New Metabolites from the South China Sea Sponge Diacarnus megaspinorhabdosa." Chemical and Pharmaceutical Bulletin 63(6): 438-442.

- Wei-Hua Jiao, Guo-Hua Shi, Ting-Ting Xu, Guo-Dong Chen, Bin-Bin Gu, Zhuo Wang, Shuang Peng, Shu-Ping Wang, Jia Li and Bing-Nan Han (2016). "Dysiherbols A–C and Dysideanone E, Cytotoxic and NF-κB Inhibitory Tetracyclic Meroterpenes from a *Dysidea* sp. Marine Sponge." *Journal of Natural Products* **79**(2): 406-411.

Presentations

- ASMR SA Scientific Meeting 6th June 2012. Adelaide, Australia. 'Investigation of anti-cancer properties of compounds extracted from South Australian Marine Sponges'. (Oral)

- AMSA-NZMSS conference, 1-5th July 2012, Hobart, Australian, 'Screening South Australian marine sponges for anti-cancer properties in vitro'.(Poster)

- AMSA-SA meeting, 12th October 2012, Adelaide, Australia 'Cytotoxic effect of extracts from South Australian marine sponges'. (Poster)

- 11th Marine Drugs Annual Meeting, 18-20th October 2013, Haikou, China, 'Cytotoxic effect of compounds from South Australian marine sponges'. (Poster)

- 9th world sponge conference, 4-8th November 2013, Fremantle, Australia, 'Screening extracts from South Australian marine sponges for cytotoxicity properties against three human breast cancer cell lines'. (Oral)

- 28th International Symposium on the Chemistry of Natural Products and 8th International Conference on Biodiversity, 19-24th October, Shanghai, China. 'Diterpenoids from South Australian marine sponges'. (Poster)

Professional Membership

- 2011-current AusBiotech (Australia's Biotechnology Organisation)
- 2012-current Australian Society for Medical Research
- 2013-current Bioprocessing Network

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Chapter 1: Introduction and Literature Review

1.1Breast cancer

1.1.1 Human breast

The human breast is an area of skin and tissue overlying the chest muscles. Females' breast is made of 15-20 modified sweat glands that produce milk (glandular tissue) as well as fatty tissue. The size of the breast is based on the amount of fat (Jatoi and Kaufmann, 2010). The structure of the healthy breast is shown in Figure 1.1. Adult female breast contains approximately 20 lobules (milk-producing glands), ducts (tiny tubes that link the lobules to the nipple), and stroma.

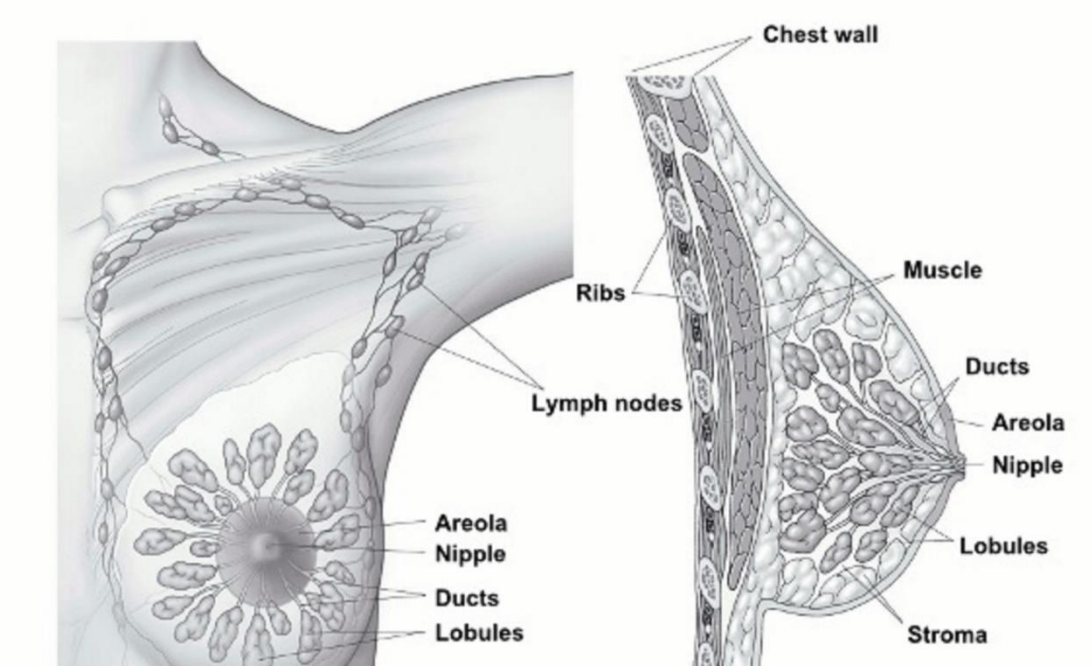


Figure 1.1: Structure of the breast

Note: The figure on the normal structure of breast contained lobules (milk-producing glands), ducts, stroma, nipple, areola, underlying pectoral muscle and ribs. This image is sourced from (http://www.cancr.net/breast-cancer/, 2016)

1.1.2 Epidemiology of breast cancer

Breast cancer is a disease in which abnormal cells in the breast tissues multiply and form a malignant tumour. Breast cancer is the most common cancer in females and second most common in the whole population in the world (Parkin, 2001, Saadat, 2008). In Australian, the risk for a female would be diagnosed with breast cancer before the age of 85 was 1 in 8, approximately 14,000 women are diagnosed each year (Cancer Australian, 2012), and the number of incidence per 100,000 population was 85 in 2012 (Figure 1.2). Breast cancer was by far the most malignancy and second common cause of cancer death in female (AIHW, 2012).

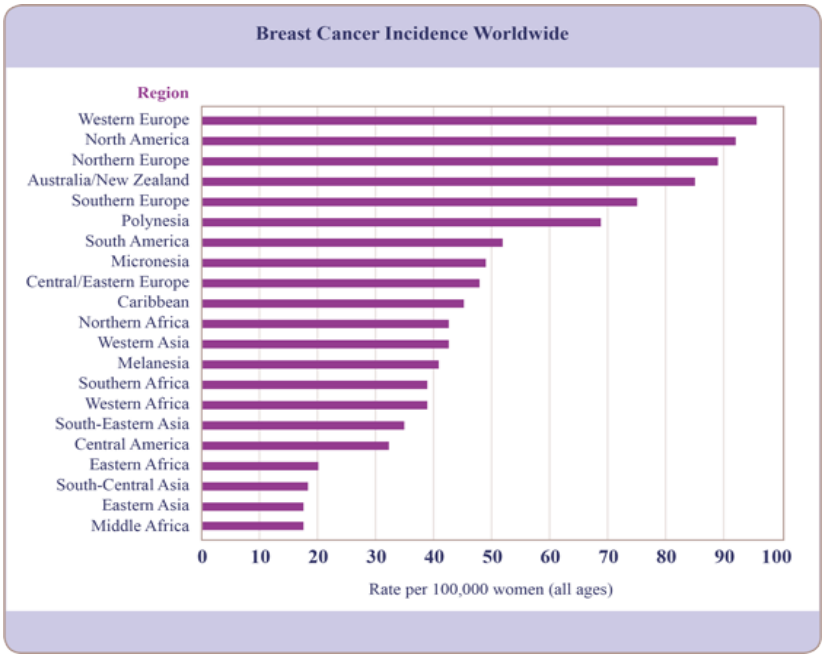


Figure 1.2: Breast cancer incidence rate worldwide

Note: The figure shows the risk rate of breast cancer per 100,000 in female in 21 difference regions in 2012. The image is sourced from (http://ww5.komen.org/BreastCancer/BreastFactsReferences.html, 2016)

1.1.3 The classification of breast cancer

Breast cancer is a malignant tumour which starts in the cells of the breast. There are several ways to categorised breast cancers by different grading systems. Based on TNM system (the size of **T**umour, whether or not spread to lymph **N**odes, and whether or not distant **M**etastasized), barest cancers are classified to stage 0 to stage 4 (Singletary et al., 2002, Edge and Compton, 2010). Breast cancers can also be categorised as ductal or lobular carcinoma based on their histological type (Li et al., 2005). Breast cancer can be further classified depending on whether cancer cells have hormone receptors (oestrogen receptor ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2). Cancer cells may have neither, one, two or all these receptors (Bauer et al., 2007, Weinberg, 2013).

1.1.4 Current therapies for breast cancer

In these days, there are several treatments for breast cancer based on its type and stage. Surgery and Radiation therapy are only targeted on the tumour without affecting the rest of the body; therefore, they are called local treatments. In contrast, systemic treatments, used to effect on the body or blood system, include chemotherapy, hormone therapy and Targeted therapy (Smith, 2006, Priestman, 2013).

Surgery is the oldest therapy for breast cancer, and it involves removing the primary tumour away from the breast. Patients can choose breast-conserving surgery or mastectomy in the early stage of breast cancer. Clinical studies showed bilateral prophylactic mastectomy reduces the risk of breast cancer approximately 90% in women with *BRCA1/2* mutations; however, the disadvantages of mastectomy includes permanent loss the one or both breasts and longer recuperation time (Grann et al., 1998,

Meijers-Heijboer et al., 2001, Rebbeck et al., 2004). After breast-conserving surgery or mastectomy, a radiation therapy may be required, especially for breast conserving surgery.

Radiation therapy uses high-energy rays or particles to destroy cancer cells. Radiation therapy gives help to reduce the growth of cancer or kill cancer cells which come back in the breast or lymph nodes after surgery. Radiation therapy may cause some side effects such as short-time skin changes, swelling or heaviness in the breast, fatigue and nausea (Hamberger, 2009, Cancer Australian, 2012).

Chemotherapy has made significant progress in both early stage and advanced breast cancer in the last decade. Patients may need a chemotherapy after surgery (adjuvant chemotherapy), before the surgery (neoadjuvant chemotherapy), or advanced breast cancer (Henderson, 1983, Hassan et al., 2010). The anthracyclines were the class of anti-breast cancer drugs since the first two anthracyclines were developed in the 1960s (Weiss, 1992). Although analysis indicated that the addition of an anthracycline to a chemotherapeutic regimen can improve the overall survival, the use of anthracyclines did, in fact, increase gastro-intestinal toxicity, cardiotoxicity and alopecia (Fossati et al., 1998, Cocconi, 2000). For patients who failed response to anthracyclines, taxane-based treatment is currently the standard of care. A combination therapy becomes increasing common in breast cancer treatment; however, taxane-anthracycline combination therapy did not improve survival in mastectomy breast cancer (Biganzoli et al., 2002). Furthermore, pre-clinical studies have demonstrated the interaction between capecitabine and taxane has been indicated in *vivo* xenograft models (Sawada et al., 1998, Kurosumi et al., 2000).

Hormone therapy can be used on patient who had hormone receptor-positive breast cancer (ER-positive or PR-positive). The aim of hormone therapy is to block and stop estrogen from attaching to these receptors of cancer cells (Jordan and Furr, 2009). There are two types of hormone therapy drugs commonly used for breast cancer. One type of hormone treatment drug is aim to stop estrogen from affecting breast cancer cells, such as tamoxifen and fulvestrant (Howell et al., 2002). The other type is aromatase inhibitors which are used to lower the estrogen levels. The aromatase inhibitors are not suitable for the premenopausal patient (Smith and Dowsett, 2003). Clinical trials showed that hormone therapy can reduce the risk of breast cancer recurrence for women who took drugs for five years, and this effect will last well beyond five years (Early Breast Cancer Trialists' Collaborative, 2005). However, hot flashes, night sweats, fatigue, vaginal symptoms, thinning hair and nails are the common side effect of hormone therapy (Young et al., 2008).

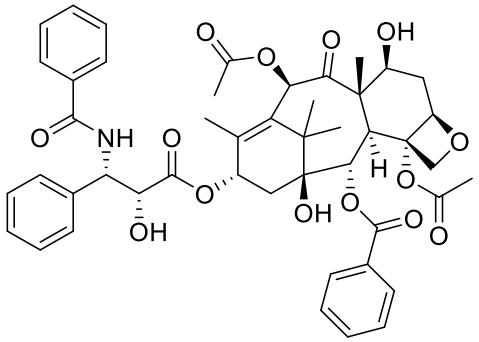
Targeted therapy drugs are designed against certain types of cancer cells, instead of against all rapidly-dividing cells (like chemotherapy). Targeted therapy can target on HER2-positive or hormone receptor-positive breast cancers (Mohamed et al., 2013). There are several drugs that have been discovered to target on growth-promoting protein (HER2) such as trastuzumab, pertuzumab, ado-trastuzumab emtansine and lapatinib. Based on the clinical trials, trastuzumab combined with standard chemotherapy can increase median survival for women who have advanced breast cancers (Nahta et al., 2006). Palbociclib and everolimus are common targeted therapy drugs used for advanced hormone receptor-positive, HER2-negative breast cancer. Despite targeted therapy drugs can make hormone therapy even more efficient, they still bring more side effects, such as low red blood cell counts, hair loss, and diarrhoea

(Tripathy, 2005). Current therapy for breast cancer are moving toward nontoxicity. Furthermore, in the future, potent targeted therapies can be customised to an individual patient's tumour (Higgins et al., 2011).

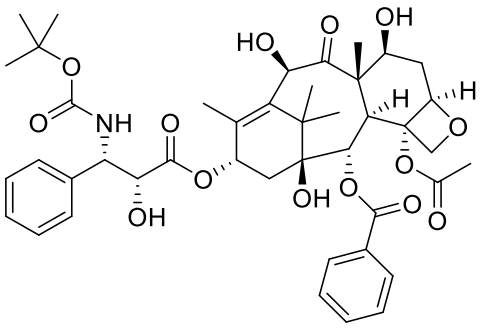
1.1.5 The drugs discovery for breast cancer

In this section, we reviewed the information on the treatments of metastatic breast cancer (MBC) were approved by US Food and Drugs Administration (FDA). From 1994 to 2014, there are 12 new drugs approved by FDA as monotherapy or combination therapy: paclitaxel, docetaxel, trastuzumab, capecitabine, gemcitabine, albumin-bound paclitaxel, lapatinib, ixabepilone, bevacizumab, eribulin, pertuzumab, and Ado-trastuzumab emtansine. However, FDA revoked the approval for bervacizumab in breast cancer after three years of its approval.

Paclitaxel (Taxol) was isolated from Pacific yew tree, *Taxus-brevifolia*, as taxol with potent anti-leukemic and tumour inhibitory properties (Wani et al., 1971). In 1994, paclitaxel received regular approval (RA) for metastatic breast cancer (MBC) from FDA. This is the first drug approved for MBC treatment by FDA since 1975. The evidence showed that 67% of patients had received prior anthracyclines in a randomized controlled trials (RCT) in 472 patients with MBC, who had failed to respond to previous anthracycline therapy, who received at a dose of 175 or 135 mg/m^2 every three week. A significant different of time to progression (TTP) was observed in the paclitaxel higher-dose arm, with a median TTP of 4.2 versus 3.0 months (hazard ratio, 0.75; $P = 0.027$) (Nabholtz et al., 1996).



Paclitaxel



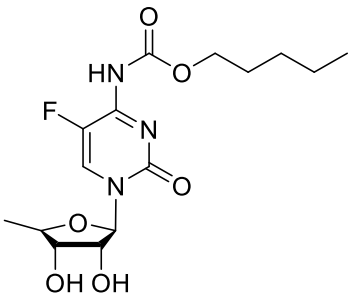
Docetaxel

Docetaxel is a derivative of taxol (Murata et al., 1994). In 1996, FDA approved docetaxel (accelerated approval) as a treatment in MBC based on six signal-arm conducted in a total 309 patients, with anthracycline-resistant MBC, and the objective response rate was 37.9% (Van Oosterom, 1995). In 1998, docetaxel received regular approved from FDA based on an RCT of 392 patients, who randomly received either docetaxel 100 mg/m^2 intravenously every 3 weeks or to the combination of mitomycin 12 mg/m^2 intravenously every 6 weeks plus vinblastine 6 mg/m^2 intravenously every 3 weeks. A significant longer in TTP for patients treated with docetaxel, with a median of 4.3 versus 2.5 months (hazard ration, 0.75; $P = 0.01$), was the basis for AA (Nabholtz et al., 1999).

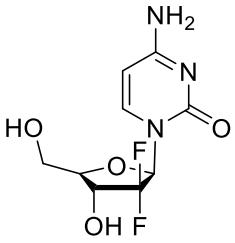
Trastuzumab received RA in 1998 as a single agent for the treatment of breast cancer as a monoclonal antibody that interferes with the HER2 receptor. In a single-agent SAT of 222 patients with HER2-overexpressing MBC, an objective response rate of 14% (2% complete response rate) was the basis for its AA (Cobleigh et al., 1999).

Capecitabine is a prodrug that is enzymatically converted to 5-fluorouracil in the body (Miwa et al., 1998). Capecitabine received accelerated approval in 1998, as a single-agent for the treatment of MBC resistant to both paclitaxel and anthracycline-containing

regimens, based on an objective response rate of 25.6% in an STA of 162 patients with refractory breast cancer (Blum et al., 1999). Three years later, FDA granted the regular approval for capecitabine using in combination with docetaxel for the treatment of MBC progressing. In a trial of 511 patients, a statistically significant TTP prolonged (hazard rate, 0.64; 95% CI, 0.54 – 0.77; $P < 0.001$) (O'Shaughnessy et al., 2002).



Capecitabine



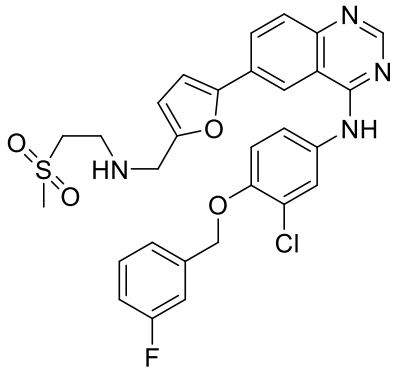
Gemcitabine

Gemcitabine was initially synthesized as a potential antiviral agent; however, it proved to have anti-tumour activity (Hertel et al., 1990). Gemcitabine received regular approval from FDA in 2004. Approval of gemcitabine was based on the RCT enrolled 529 patients with locally advanced or MBC, who were randomly assigned to receive gemcitabine 1,250mg/m^2 on day 1 and 8 with paclitaxel 175mg/m^2 on day 1 or to single-agent paclitaxel 175 mg/m^2 on a 21-day cycle. A significant longer median TTP in the patients treated with the gemcitabine plus paclitaxel combination compared to that treated with paclitaxel alone, with 5.2 months versus 2.9 months (hazard rate, 0.65; 95% CI, 0.52 – 0.81; $P < 0.001$) (Albain et al., 2008).

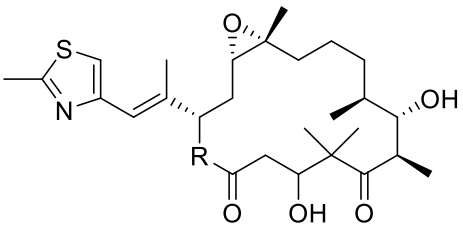
Despite the wide application of paclitaxel in clinic, it is associated with significant toxicities including nail changes, fatigue, alopecia, myelosuppression and neuropathy. To improve the therapeutic index of paclitaxel, it was bound to nanoparticles of the

naturally occurring vehicle for hydrophobic molecules, albumin (Robinson and Keating, 2006). Albumin-bound paclitaxel is a paclitaxel formulation approved by FDA in 2005. The approval was based on an RCT in 460 patients with progressive MBC after prior anthracycline-containing chemotherapy regimens and a significant higher objective response rate compared with paclitaxel (21.5%; 95% CI, 16.2 – 26.7 versus 11.1%; 95% CI, 6.9 – 15.1) (Gradishar et al., 2005).

Lapatinib is a tyrosine kinase inhibitor, inhibits epidermal growth factor receptor type 1 (EGFR) and HER2, synthesized in 1999 (Wood et al., 2004). Lapatinib was approved by FDA for use in combination with capecitabine in MBC patients with HER2-overexpressing. The approval was based on an RCT of 399 patients overexpressing HER2, and a significant TTP improvement was observed that median difference in TTP based on independent radiology review was 8.5 weeks (hazard rate, 0.57; 95% CI, 0.43 – 0.77; $P < 0.001$) and 5.6 weeks based on investigator assessment (hazard rate, 0.72; 95% CI, 0.56 – 0.92; $P < 0.0076$) (Cameron et al., 2008, Geyer et al., 2006).



Lapatiib



Epothilone B: R=O
Ixabepilone: R=NH

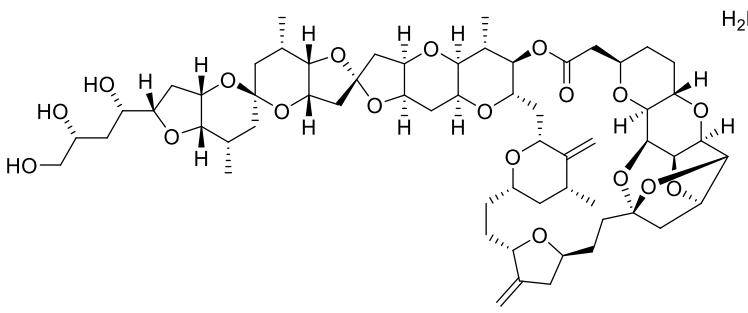
Ixabepilone, developed by Bristol-Myers Squibb Company, is a semisynthetic analogue of natural product epothilone B (Lee et al., 2001). Ixabepilone received regular approval

in MBC for both monotherapy and combination therapy with capecitabine in 2007. For monotherapy approval, the objective response rate was 12.4% by independent radiology review (95% CI, 6.9% - 19.9%) and 18.3% by investigator assessment (95% CI, 11.9% - 26.1%) in a multicentre SAT in 126 patients with MBC, who had two or more chemotherapy treatments. The combination therapy approval was based on an RCT of 572 patients with MBC, who had pre-treated or resistant to anthracyclines and resistant to taxane. Patients have randomly received either the combination of Ixabepilone 40 mg/m^2 intravenously every 21 days and capecitabine monotherapy 1,250 mg/m^2 twice per day for 14 days. A statistically significant improvement in median progression-free survival (5.7 months versus 4.1 months, hazard rate, 0.69; 95% CI, 0.58 – 0.83; $P < 0.001$) was a basis for its approval (Thomas et al., 2007).

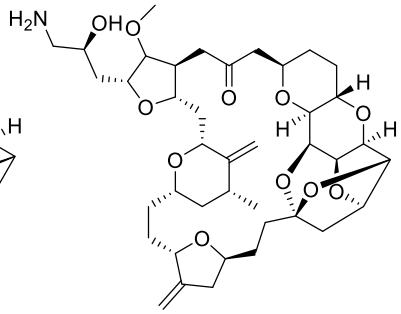
Bevacizumab is a recombinant humanized monoclonal antibody, has high affinities for vascular endothelial growth factor (Kim et al., 1992). In 2008, bevacizumab received accelerated approval as combination therapy with paclitaxel for patients who have not received chemotherapy for metastatic HER2-negative breast cancer. This approval was supported by an RCT of 722 patients, who were randomly assigned to receive either bevacizumab combination with paclitaxel (10 mg/kg intravenously every two weeks) or paclitaxel alone (90 mg/m2 intravenously weeks 1 to 3 of each four-week cycle). A significant improvement in median progression-free survival, 11.3 months (combination arm) versus 5.8 months (paclitaxel alone arm) (hazard rate, 0.48; 95% CI, 0.39 – 0.61; $P < 0.001$) was basis for this approval (Gray et al., 2009, Miller et al., 2007). However, FDA cancelled the approval of bevacizumab in MBC as a review of results that bevacizumab did not extended the lives of women with metastatic breast cancer and the patients showed increased risk of side effects such as bleeding, heart

attack, and heart failure. However, the drug is still approved for treatment in renal cancer, colorectal cancer and lung cancer (Tanne, 2011).

Eribulin is a synthetic macrocyclic analogue of the marine natural product halichondrin B, isolated from marine sponge *Halichondria okadai* and was reported to show anti-tumour activities (Hirata and Uemura, 1986). Eribulin was granted regular approval by FDA in 2010 for the treatment of patients with MBC, who have pre-treatment with taxane, anthracycline and at least two chemotherapeutic regimens. The approval for eribulin was based on an improvement in overall survival, with median overall survival of 13.1 months for eribulin-treated patients versus 10.6 months for patients treated with other drugs (vinorelbine 24%, gemcitabine 18%, capecitabine 17%, taxane 16%, anthracycline 9%, hormone therapy 4%, and miscellaneous other agents 10%) (Hazard rate, 0.81; 95% CI, 0.66 – 0.99; $P < 0.041$) in an RCT of 762 patients with MBC who had pre-treated with at least two chemotherapeutic regimens (Cortes et al., 2011).



Halichodrin B



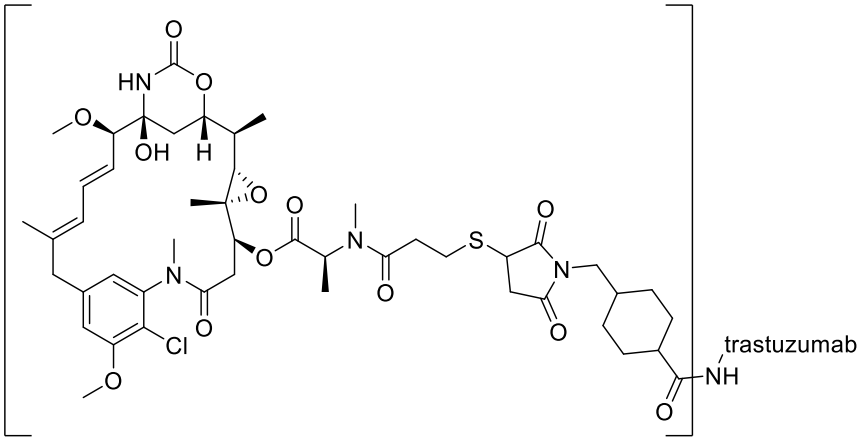
Eribulin

Pertuzumab is a monoclonal antibody, targeting the HER2 receptor extracellular domain. In 2012, the FDA approved pertuzumab for use in combination with trastuzumab and docetaxel in MBC for patients who show HER2-positive but have not

received prior anto-HER2 therapy of chemotherapy (Blumenthal et al., 2013). This approval was the first time for FDA approved a monoclonal antibody to use in combination therapy with another monoclonal antibody. This approval was based on a phase III clinical trial with 808 patients who have HER2-posivatie MBC. Those patients randomly received (1:1) pertuzumab (n = 402) or placebo (n = 406) in combination therapy with trastuzumab and docetaxel. A significant improvement in median progression-free survival, 18.5 months (pertuzumab arm) versus 12.4 months (placebo arm) (hazard rate, 0.62; 95% CI, 0.51 – 0.75; $P < 0.0001$) was the basis for this approval (Baselga et al., 2012). However, FDA noticed the applicant did not ha a consistent process to ensure continued supply of commercial material in 2012, the approval only allowed the drug product from 2010 manufacturing into the market (Blumenthal et al., 2013).

Ado-trastuzumab emtansine (in the United States) is an antibody-drug consisted conjugate consisted of monoclonal antibody trastuzumab and cytotoxic agent emtansine (LoRusso et al., 2011). In 2013, the FDA approved ado-trastuzumab emtansine for use as a single drug for the treatment in MBC of patients who have HER2-positive and previously received trastuzumab and a taxane treatment separately or in combination. This approval was based on a phase III trial of 991 patients with HER2-positive MBC. These patients randomly received (1:1) ado-trastuzumab emtansine (n = 495) or lapatinib in combination with capecitabine (n = 496). Statistically significant improvements in median progression-free survival, 9.6 months (ado-trastuzumab emtansine arm) versus 6.4 months (combination arm) (hazard rate, 0.65; 95% CI, 0.55 – 0.77; $P < 0.0001$), and overall survival, 30.9 months (ado-trastuzumab emtansine arm)

versus 25.1 months (combination arm) (hazard rate, 0.68; 95% CI, 0.55 – 0.85; $P < 0.001$) were the basis for this approval (Verma et al., 2012).



Trastuzumab emtansine

A summary of time line of breast cancer treatments were approved by FDA as shown in Figure 1.3. Despite there are 12 news drugs have been development in breast cancer treatment since 1994, current therapeutic strategies indicated limitations and side effects, such as flashes, night sweats, thinning hair, low red blood cell counts, and diarrhoea (Fossati et al., 1998, Cocconi, 2000, Tripathy, 2005, Young et al., 2008), and they are not satisfactory due to chemo-resistance. Therefore, the identification of new treatment for breast cancer remains a high priority.

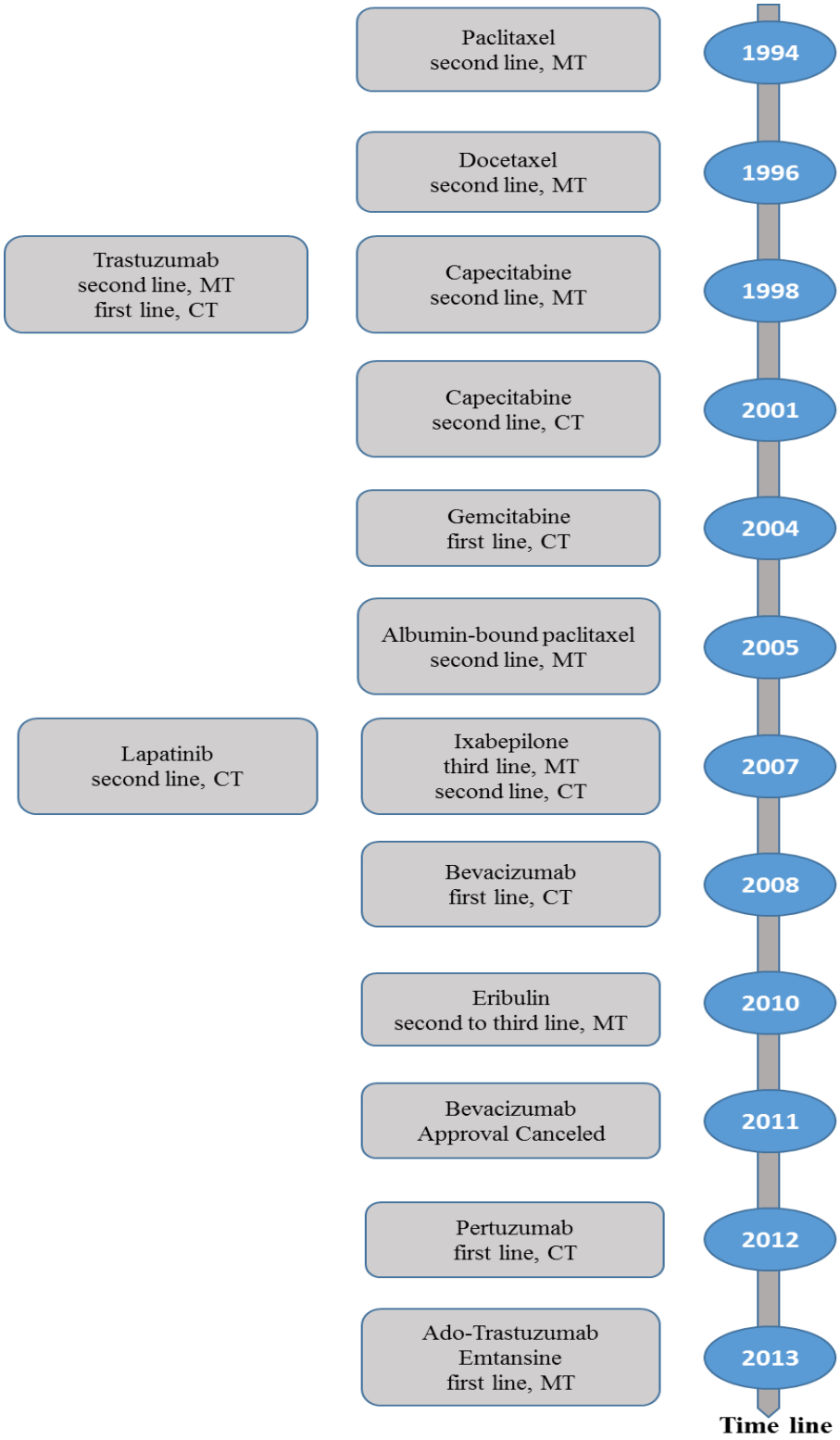


Figure 1.3: FDA approved breast cancer treatment time line

Note: Monotherapy (MT), Combination therapy (CT).

1.2Marine Natural Products

1.2.1 Drugs from the Sea

Natural products from terrestrial plants and microbes have long been a traditional source of drug discovery; however, the oceans cover approximately 70% of the earth's surface, and marine species contains nearly 50% of total biodiversity found on Earth (Thakur et al., 2005). That was because the lack of technology (like scuba diving) of collect the source organisms. In 1950s, several unusual *arabino*- and *ribo*-pentosyl nucleosides were obtained from marine sponges (collected from Florida, USA), and those compounds eventually led to the development of two significant anticancer nucleosides that have been in clinical use for decades (Bergmann and Burke, 1956, Bergmann and Feeney, 1951, Bergmann and Stempien, 1957). However, before 1985, there were less than 100 novel natural products obtained from marine resources annually. In 1987, the number of new marine natural products was increased to over 300 and remained at a range of 400-600 products per year in the 1990s. There were more than 1000 new marine natural products reported in the literature in 2008. Since then, the marine natural products entered into the era with annual discovered of over 1000 new compounds (Figure 1.4).

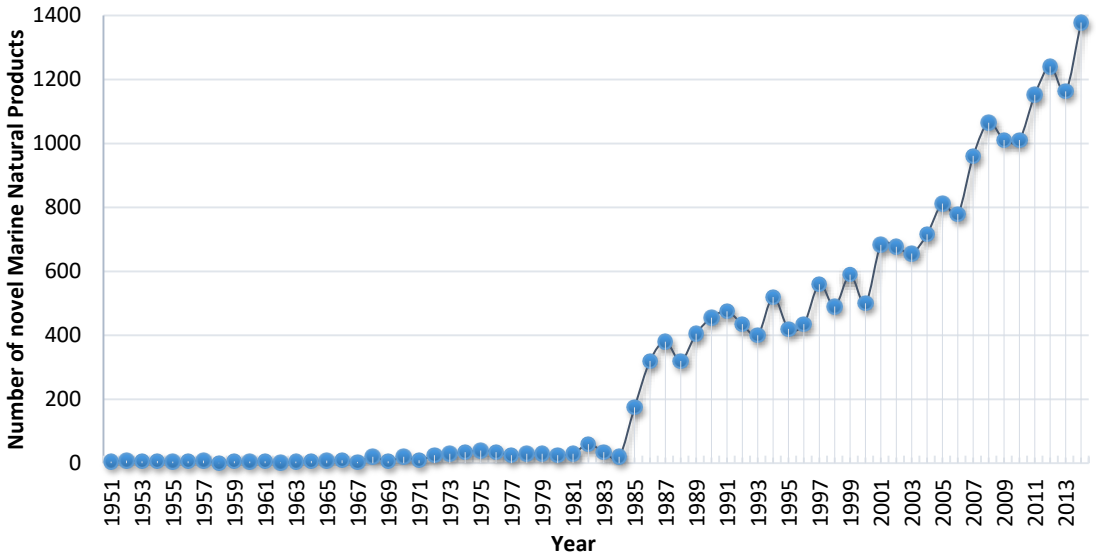


Figure 1.4: Temporal trend in the number of novel compounds isolated form marine organisms from 1951 to 2013.

Sources: (Blunt et al., 2007, Blunt et al., 2008, Blunt et al., 2009, Blunt et al., 2015, Blunt et al., 2012, Blunt et al., 2013, Blunt et al., 2014, Blunt et al., 2016, Blunt et al., 2003, Blunt et al., 2004, Blunt et al., 2005, Blunt et al., 2006, Blunt et al., 2010, Blunt et al., 2011, Faulkner, 1984b, Faulkner, 1984a, Faulkner, 1986, Faulkner, 1987, Faulkner, 1988, Faulkner, 1990, Faulkner, 1991, Faulkner, 1992, Faulkner, 1993, Faulkner, 1994, Faulkner, 1995, Faulkner, 1996, Faulkner, 1997, Faulkner, 1998, Faulkner, 1999, Faulkner, 2000, Faulkner, 2001, Faulkner, 2002, Jha and Zi-Rong, 2004, Proksch et al., 2003, Proksch et al., 2002)

With this impressive number of marine-derived novel compounds discovered, a number of new drugs from marine natural products have been approved for markets and clinical trials. To date, two marine natural products (Ziconotide and trabectedin) became therapeutic agents, and five other synthetic therapeutic agents (Cytarabine, vidarabine, Omega-3-acid ethyl esters, eribulin mesylate, brentuximab, and vedotin) was derived from the chemical structures of marine products. In additional, over 20 potential therapeutic agents from marine sources are in clinical trials (phase I, II, or III), and most of the agents target on the disease of cancers (Table 1.1).

Table 1.1: Marine natural products and derivatives in clinical development

Clinical Status	Compound Name	Chemical Class	Collected Source Organism	Disease Area
FDA Approved	Cytarabine (Ara-C)	Nucleoside	Sponge	Cancer Leukaemia
	Vidarabine (Ara-A)	Nucleoside	Sponge	Antiviral
	Ziconotide	Peptide	Cone snail	Pain
	Omega-3-acid ethyl esters	Omega-3 fatty acides	Fish	Hypertriglyceridemia
	Eribulin Mesylate	Macrolide	Sponge	Cancer Metastatic breast cancer
	Brentuximab vedotin	ADC (MMAE)	Mollusc	Cancer
	Trabectedin	Alkaloid	Tunicate	Cancer
Phase III	Plinabulin	Diketopiperazine	Fungus	Cancer
	Plitidepsin	Desipeptide	Tunicate	Cancer
	Tetrodotoxin	Guanidinium alkaloid	Pufferfish	Pain
Phase II	ABT-414 EGFRvIII-MMAF	ADC (MMAF)	Mollusc/ cyanobacterium	Cancer
	DMXBA	Alkaloid	Worm	Cognition, Schizophrenia
	Glembatumum ab Vedotin	ADC (MMAE)	Mollusc/ cyanobacterium	Cancer
	Lurbinectedin	Alkaloid	Tunicate	Cancer

Phase II	AGS-16C3F	ADC (MMAF)	Mollusc/ cyanobacterium	Cancer
	Lifastuzumab vedotin DNIB0600A	ADC (MMAE)	Mollusc/ cyanobacterium	Cancer
	Pinatuzumab vedotin	ADC (MMAE)	Mollusc/ cyanobacterium	Cancer
	Polatuzumab vedotin	ADC (MMAE)	Mollusc/ cyanobacterium	Cancer
	GSK2857916	ADC (MMAF)	Mollusc/ cyanobacterium	Cancer
	ASG-67E	ADC (MMAE)	Mollusc/ cyanobacterium	Cancer
	ASG-15ME	ADC (MMAE)	Mollusc/ cyanobacterium	Cancer
	ASG-22ME	ADC (MMAE)	Mollusc/ cyanobacterium	Cancer
Phase I	Bryostatin	Macrolide Lactone	Bryozoan	Cancer
	DSTP3086S	ADC (MMAE)	Mollusc/ cyanobacterium	Cancer
	HuMas-TF-ADC	ADC (MMAE)	Mollusc/ cyanobacterium	Cancer
	Marizomib	Beta-lactone-gamma lactam	Bacterium	Cancer
	MLN-0264	ADC (MMAE)	Mollusc/ cyanobacterium	Cancer
	PM060184	Polyketide	Sponge	Cancer
	SGN-CD19A	ADC (MMAF)	Mollusc/ cyanobacterium	Cancer
	SGN-LIV1A	ADC (MMAE)	Mollusc/ cyanobacterium	Cancer

Note: FDA: Food and Drug Administration (USA); ADC: Antibody Drug Conjugate; AAME: Monomethylauristatin E; AAMF: Monomethylauristatin F. Sources: (Gerwick and Moore, 2012, Mayer et al., 2010, Simmons et al., 2005, McLaughlin, 2015)

1.2.2 The role of marine sponges in marine natural product discovery

Marine sponge (phylum: Porifera) is the oldest multicellular animal, which has an approximately 700 to 800 million years living history in this planet (Becerro, 2012, Müller, 2012). Sponge is the richest source of novel secondary metabolites in marine natural products discovery. There were 26,040 novel compounds isolated from marine organisms from 1963 to 2014, and 33.1% (8435) of these new compounds are obtained from marine sponges. For marine sponges, 285 genera had been studied for their chemical constituent, and the most studied genus is *Dysidea* from which 2,991 novel compounds were isolated (Blunt et al., 2016, Blunt et al., 2015).

Over 200 new compounds have been isolated from marine sponges annually from 2001 to 2014, especially, 355 novel compounds were purified from sponges in 2012 (Marine Natural products, 2003 to 2016). Even through the percentage of new compounds from sponges in the whole novel compounds discovery decreased annually, there were still approximately 20% of new compounds isolated from marine sponges except the year 2010 (Figure 1.5).

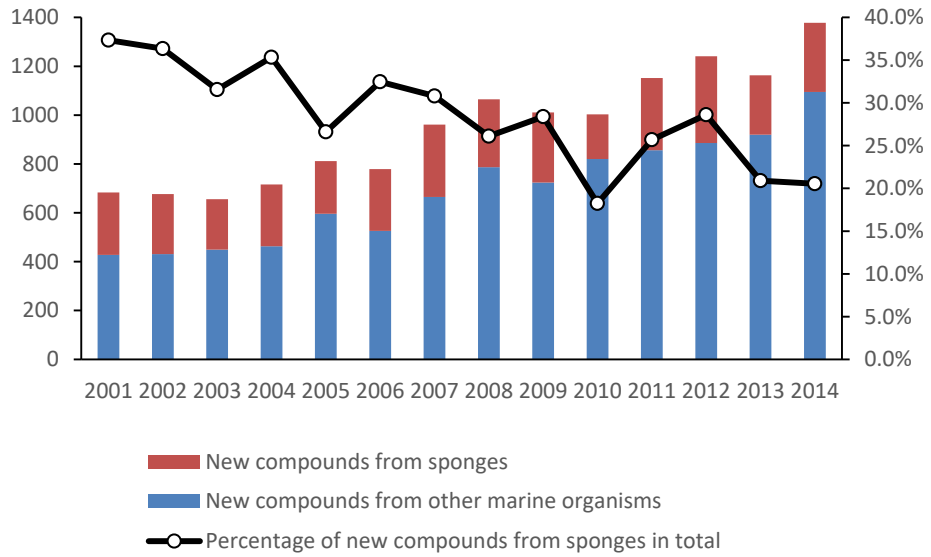


Figure 1.5: The contribution of novel compounds isolated from marine sponges

Sources: (Blunt et al., 2003, Blunt et al., 2016, Blunt et al., 2007, Blunt et al., 2006, Blunt et al., 2014, Blunt et al., 2004, Blunt et al., 2009, Blunt et al., 2008, Blunt et al., 2005, Blunt et al., 2010, Blunt et al., 2013, Blunt et al., 2012, Blunt et al., 2011, Blunt et al., 2015)

With regard to the bioactivity of compounds isolated from marine resources; there are mainly eight types of biological activity reported: anticancer, anti-bacteria, anti-fungus, anti-virus, pest resistance, prevention of heart and vascular disease, protection of neurons/neurotoxicity, and other activities. The major bioactive metabolites from marine sponges account for 36.73% of total bioactive compounds from 1985 to 2012. Additionally, over 30% of compounds isolated from sponges indicated bioactivities, and more than 50% of those bioactive metabolites showed anti-cancer activity (Figure 1.6).

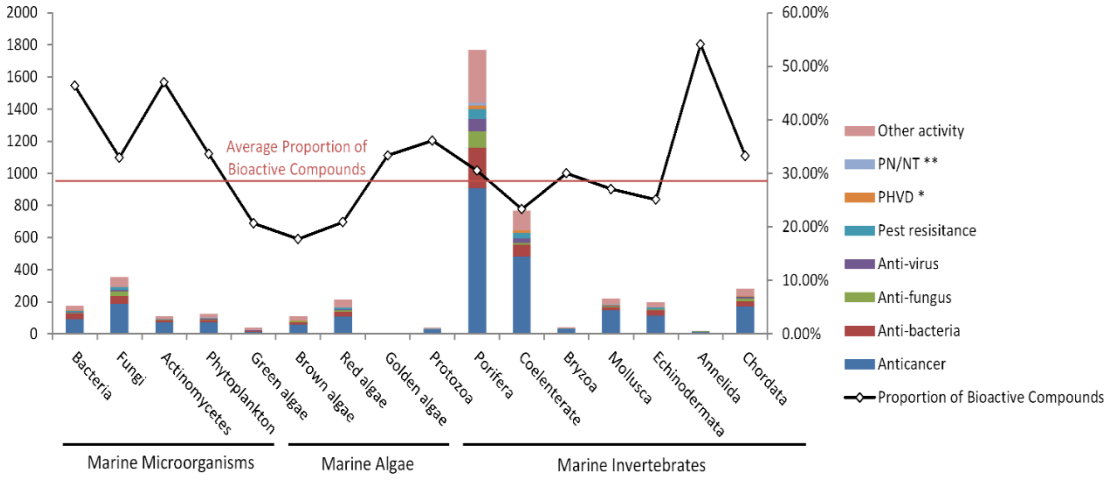


Figure 1.6: Number and proportion of bioactive/non-bioactive compounds from marine organisms (∗ PHVD: prevention of heart and vascular disease, ∗∗ PN/NT: Protection of neurons/neurotoxicity)

Source: (Hu et al., 2015)

1.2.3 The cytotoxicity of compounds from marine sponges against breast cancer cell lines (2001 -2010)

From 2001 to 2010, there are 37 articles reporting 101 secondary metabolites from marine sponges with cytotoxicity against breast cancer cell lines, isolated from marine sponges. 37 sponge samples (31 sponge genera) were described in these articles. Four human breast cancer cell lines (MCF7, MDA-MB-435, MDA-MB-231, and T47D) have been used for cytotoxicity tests, and MDA-MB-231 and MCF7 are the most common cell lines as the model cell lines to test cytotoxicity of novel marine compounds (Table 1.2). The value of IC_{50} and GI_{50} are the most common index to quantify the cytotoxicity of compounds. All those compounds have IC_{50}/GI_{50} lower than 50 μg/ml. The most cytotoxic compounds against breast cancer cell line are irciniastatins A and B isolated from sponge *Ircinia ramose*, and showed cytotoxicity against MCF7 cells with GI_{50} value of 0.0032 μg/ml and 0.0005 μg/ml, respectively (Pettit et al., 2004b).

Table 1.2: Statistics of compounds (cytotoxicity against human breast cancer cell lines) isolated from marine sponges (2001 -2010)

	Number		Number
Articles	37	Novel compounds	101
Sponge samples	37	Sponge genera	31
The times of human breast cancer cell lines used in cytotoxicity studies			
MCF7	16	MDA-MB-435	2
MDA-MB-231	16	T47D	5

Note: The resources for this 10 years statistics from (Blunt et al., 2011, Blunt et al., 2012, Blunt et al., 2010, Blunt et al., 2005, Blunt et al., 2008, Blunt et al., 2009, Blunt et al., 2004, Blunt et al., 2006, Blunt et al., 2007, Blunt et al., 2003, Inman et al., 2010, Wei et al., 2010, Holland et al., 2009, Tung et al., 2009a, Tung et al., 2009b, Yao et al., 2009, El-Naggar and Capon, 2009, Laville et al., 2009, Coello et al., 2009, Zampella et al., 2009, Mao et al., 2009, Ankudey et al., 2008, Capon et al., 2008, Hernández-Guerrero et al., 2007, Reyes et al., 2007, Dai et al., 2007, Diaz-Marrero et al., 2006, Dai et al., 2006, Hernández-Guerrero et al., 2006, Sorek et al., 2006, Martín et al., 2005, Granato et al., 2005, Berrué et al., 2005, Charan† et al., 2004, Pettit et al., 2004b, Sonnenschein et al., 2004, Huang et al., 2004, Wonganuchitmeta et al., 2004, Pettit et al., 2004a, Charan et al., 2002a, Charan et al., 2002b, Erickson et al., 2003, Charan et al., 2001)

1.2.4 Breast cancer cell lines

The first breast cancer cell line was BT-20 established in 1958, and the most commonly used breast cancer cell line is MCF-7 established in 1973 due to its exquisite hormone sensitivity through the expression of ER (Levenson and Jordan, 1997, Soule et al., 1973, Lasfargues and Ozzello, 1958). There are several ways to classify human breast cancer cell lines. Basal cells and luminal epithelial cells are the two distinct types of epithelial cell in human mammary gland (Perou et al., 2000). Based on the status of receptors, breast cancer cells can be classified as ER (oestrogen receptor)-positive/negative; PR (progesterone receptor)-positive/negative and HER2 (human epidermal growth factor receptor 2)-positive (overexpression). Also, breast cancer cells can be categorised based on histological type, tumour grade and lymph node status (Holliday and Speirs, 2011). The different subtype of breast cancer cell line has different prognosis and treatment response. For example, ER is a therapeutic target, HER2 is the target for trastuzumab

therapy, and luminal subtype is a potential hormone therapy target (Sørlie et al., 2001). Table 1.3 shows the characteristics of the most common breast cancer cell lines used for cytotoxicity study.

Table 1.3: The characteristics of human breast cancer cell lines used in cytotoxicity studies

Cell line	Gene cluster	ER	PR	HER2	P53	Tumour type
MCF 7	Lu	+	[+]		$+/-^{WT}$	IDC
T47D	Lu	+	[+]		$++^{M}$	IDC
MDA-MB-231	B	–	[–]	+	$-^{M}$	AC
MDA-MB-435	B	–	[–]		$+^{WT}$	IDC

Note: Lu, luminal; B, Basal; ER/PR, + positivity; HER2, + overexpression; P53, protein levels and mutational status, ^{M}, mutant protein; ^{WT}, wild-type protein; AC, adenocarcinoma; IDC, invasive ductal carcinoma. Source: (Neve et al., 2006)

1.2.5 Marine sponges from Australia and South Australia

The marine territory of Australia is approximately 9.0 million km^2. There are over 1,500 sponge species reported so far; however, several thousand other species have been discovered and new to science (Butler et al., 2010). The distribution of Australian sponge collection sites showed in Figure 1.7, although, South Australian water was not considered as 'hotspot' for sponge collection by Butler, there still were over 1000 sponge species (200 genera) described from southern Australia (Sorokin et al., 2005). Therefore, with rich untapped marine sponges in South Australia, there is considerable potential to isolate and identify novel anti-cancer agents.

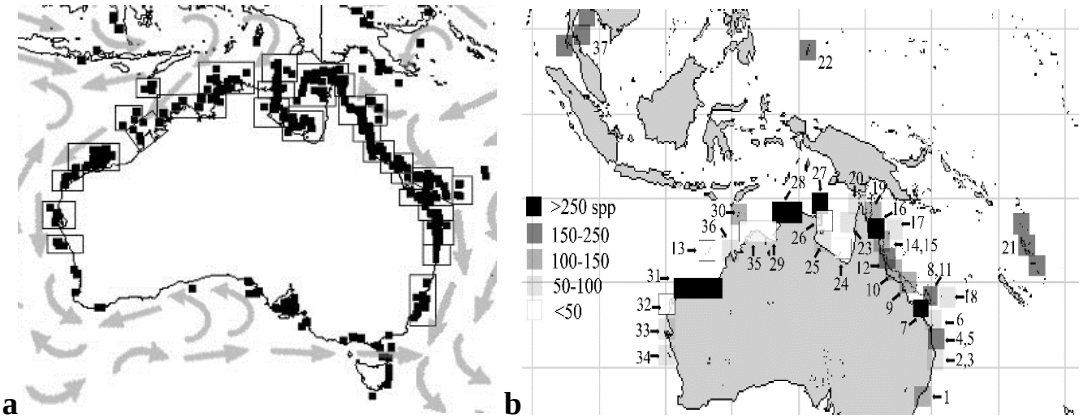


Figure 1.7: Distribution of Australian sponge collection sites

Note: a: Individual sponge collection sites in Australian; b: Species richness of sponge collection sites in Australian. Source: (Hooper et al., 2002)

1.2.6 The potent anti-cancer compounds isolated from Australian marine sponges

A review of anti-cancer compounds isolated from Australian marine sponges was shown in Table 1.4. There are total 332 new compounds have been isolated from Australian marine sponges from 1994 to 2013, and 37 novel compounds have been identified with cytotoxicity against human cancer cell lines. Importantly, 20 of these compounds indicated with an IC_{50} or GI_{50} value lower than 5 μg/ml against human cancer cell lines, and 10 compounds have IC_{50} or GI_{50} value even lower than 1 μg/ml. Unfortunately, none of these marine natural product have entered a clinical trial.

Table 1.4: Anti-cancer compounds isolated from Australian marine sponges from 1994 to2013

Compound Name	Sponge species	Chemical class	Collection location	Anti-cancer efficacy	Reference
Coscinoquinol (A1)	*Coscinoderma* sp.	Sesterterpene	Great Barrier Reef	Showed cytotoxicity against P388, A549, and HT29, (IC_{50} = 0.25, 0.5, and 0.25 μg/ml, respectively)	(Alea et al., 1994)
Chondropsins A (A2)	*Chondropsis* sp	Macrolide lactam	Bass Island, Wollongong	Showed growth inhibition of 60 tumour cell lines with GI_{50} value range of 2.4×10^{-8} to 2.4×10^{-5}M	(Cantrell et al., 2000b)
Spongiadioxins A (A2)	*Dysidea dendyi*	Tetrabromodibenzo-*p*-dioxin	Scott Reef	Showed cytotoxicity against mouse Ehrlich cancer cells with IC_{50} = 29 μg/ml.	(Utkina et al., 2001)
Spongiadioxins B (A4)	*Dysidea dendyi*	Tetrabromodibenzo-*p*-dioxin	Scott Reef	Showed cytotoxicity against mouse Ehrlich cancer cells with IC_{50} = 15.5 μg/ml.	(Utkina et al., 2001)
Leucamide A (A5)	*Leucetta microraphis*	Heptapeptide	Great Barrier Reef	Showed growth inhibition on HM02, HepG2 and Huh7, (IC50 = 5.2, 5.9, and 5.1μg/ml, respectively)	(Kehraus et al., 2002)

Zyzzyanone A (A6)	*Zyzzya fuliginosa*	Alkaloid	Great Barrier Reef	Showed cytotoxicity against mouse Ehrlich carcinoma cells with IC_{50} = 25 μg/ml	(Utkina et al., 2004)
Zyzzyanone B (A7)	*Zyzzya fuliginosa*	Alkaloid	Great Barrier Reef	Showed cytotoxicity against mouse Ehrlich carcinoma cells with IC_{50} = 25 μg/ml	(Utkina et al., 2005)
Zyzzyanone C (A8)	*Zyzzya fuliginosa*	Alkaloid	Great Barrier Reef	Showed cytotoxicity against mouse Ehrlich carcinoma cells with IC_{50} = 25 μg/ml	(Utkina et al., 2005)
Zyzzyanone D (A9)	*Zyzzya fuliginosa*	Alkaloid	Great Barrier Reef	Showed cytotoxicity against mouse Ehrlich carcinoma cells with IC_{50} = 25 μg/ml	(Utkina et al., 2005)
Trachycladindole A (A10)	*Trachycladus laevispirulifer*	Heterocycles	Great Australia Bight	Showed cytotoxicity against A549, HT29, and MDA-MB-231, (GI_{50} = 6.5, 2.9, and 1.2 μg/ml, respectively)	(Capon et al., 2008)
Trachycladindole B (A11)	*Trachycladus laevispirulifer*	Heterocycles	Great Australia Bight	Showed cytotoxicity against A549, HT29, and MDA-MB-231, (GI_{50} = 1.3, 0.5, and 2.7 μg/ml, respectively)	(Capon et al., 2008)

Trachycladindole (A12)	C	*Trachycladus laevispirulifer*	Heterocycles	Great Australia Bight	Showed cytotoxicity against A549, HT29, and MDA-MB-231, (GI_{50} = 19.8, 4.8, and 12.2 μg/ml, respectively)	(Capon et al., 2008)
Trachycladindole (A13)	D	*Trachycladus laevispirulifer*	Heterocycles	Great Australia Bight	Showed cytotoxicity against A549, HT29, and MDA-MB-231, (GI_{50} = 1.7, 0.4, and 2.4 μg/ml, respectively)	(Capon et al., 2008)
Trachycladindole (A14)	E	*Trachycladus laevispirulifer*	Heterocycles	Great Australia Bight	Showed cytotoxicity against A549, HT29, and MDA-MB-231, (GI_{50} = 0.5, 0.3, and 1.1 μg/ml, respectively)	(Capon et al., 2008)
Trachycladindole (A15)	F	*Trachycladus laevispirulifer*	Heterocycles	Great Australia Bight	Showed cytotoxicity against A549, HT29, and MDA-MB-231, (GI_{50} = 1.2, 0.8, and 2.3 μg/ml, respectively)	(Capon et al., 2008)
Bastadin 24 (A16)		*Ianthella quadrangulata*	Alkaloid	Hero Island's Wistari Reef	Showed cytotoxicity against 36 human tumour cell lines with mean IC_{50} = 1.8 μg/ml	(Greve et al., 2008)
Phorbasin E (A17)		*Phorbas* sp.	Diterpene	Great Australia Bight	Showed growth inhibition on A549, HT29 and MDA-MB-231 with GI_{50} range of 5 – 15 μM	(Zhang and Capon, 2008)

Phorbasin G (A18)	*Phorbas* sp.	Diterpene	Great Australia Bight	Showed growth inhibition on A549, HT29 and MM96L, (GI_{50} = 4.7, 3.1, and 3.1 μg/ml, respectively)	(Zhang et al., 2008)
Phorbasin H (A19)	*Phorbas* sp.	Diterpene	Great Australia Bight	Showed growth inhibition on A549, HT29 and MM96L, (GI_{50} = 2.8, 2.8, and 0.8 μg/ml, respectively)	(Zhang et al., 2008)
Phorbasin I (A20)	*Phorbas* sp.	Diterpene	Great Australia Bight	Showed growth inhibition on A549, HT29 and MM96L, (GI_{50} = 2.5, 2.5, and 1.7 μg/ml, respectively)	(Zhang et al., 2008)
Phorbasin J (A21)	*Phorbas* sp.	Diterpene	Great Australia Bight	Showed growth inhibition on A549, HT29 and MM96L, (GI_{50} = 3.9, 6.1, and 2.2 μg/ml, respectively)	(Zhang et al., 2008)
Phorbasin K (A22)	*Phorbas* sp.	Diterpene	Great Australia Bight	Showed growth inhibition on A549, HT29 and MM96L,(GI_{50} = 11.9, >29.8, and 6.0 μg/ml, respectively)	(Zhang et al., 2008)
(+)-dihydrodiscorhabdin A (A23)	Genera *Higginsia*	Alkaloid	Deal Island	Showed cytotoxicity against A549, HT29, and MDA-MB-231 with GI_{50} value range of 0.1-0.5 μg/ml	(El-Naggar and Capon, 2009)

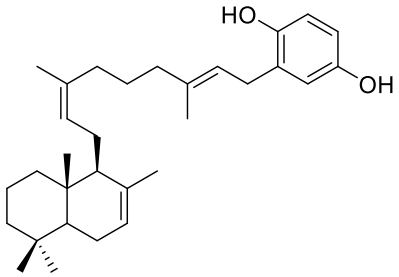
(+)-dihydrodiscorhabdin L (A24)	Genera *Spongosorites*	Alkaloid	Port Campbell	Showed cytotoxicity against A549, HT29, and MDA-MB-231 with GI_{50} value range of 0.1-0.5 μg/ml	(El-Naggar and Capon, 2009)
3α,12α,16α-Trihydroxy-24ξ-ethylcholest-25-ene (A25)	*Psammoclema* sp.	Steroid	Nelson Bay	Showed growth inhibition on HT29, MCF-7, A2780, and DU145, (GI_{50} = 7.1, 10, 11, and 13 μg/ml, respectively)	(Holland et al., 2009)
3α,12α,16α-Trihydroxy-24*R*-methylcholest-22*E*-ene (A26)	*Psammoclema* sp.	Steroid	Nelson Bay	Showed growth inhibition on HT29, MCF-7, A2780, and DU145, (GI_{50} = 7.0, 17, 10, and 27 μg/ml, respectively)	(Holland et al., 2009)
3α,12α,16α-Trihydroxy-24-methylcholest-24(28)-ene (A27)	*Psammoclema* sp.	Steroid	Nelson Bay	Showed growth inhibition on HT29, MCF-7, A2780, and DU145, (GI_{50} = 18, 18, 19, and 27 μg/ml, respectively)	(Holland et al., 2009)
3α,12α,16α-Trihydroxycholestane (A28)	*Psammoclema* sp.	Steroid	Nelson Bay	Showed growth inhibition on HT29, MCF-7, A2780, and DU145, (GI_{50} = 5.0, 5.5, 5.4, and 6.7 μg/ml, respectively)	(Holland et al., 2009)

Ecionine A (A29)	*Ecionemia geodides*	Alkaloid	Moorina Bay, Tasmania	Showed cytotoxicity against TSU-Pr1, TSU-Pr1-B1, TSU-Pr1-B2, 5637 (IC_{50} = 6.5, 6..5, 3.6, and 3.7 μg/ml, respectively)	(Barnes et al., 2010)
Franklinolide A (A30)	*Geodia* sp. With thinly *Halichondria* sp.	Polyketide	Great Australia Bight	Showed growth inhibition on AGS and HT29 (GI_{50} = 0.3, and 0.1 μg/ml, respectively)	(Zhang et al., 2010)
Franklinolide B (A31)	*Geodia* sp. With thinly *Halichondria* sp.	Polyketide	Great Australia Bight	Showed growth inhibition on AGS and HT29 (GI_{50} = 0.7, and 0.5 μg/ml, respectively)	(Zhang et al., 2010)
Franklinolide C (A32)	*Geodia* sp. With thinly *Halichondria* sp.	Polyketide	Great Australia Bight	Showed growth inhibition on AGS and HT29 (GI_{50} = 9.3, and 4.7 μg/ml, respectively)	(Zhang et al., 2010)
Metachromi V (A33)	*Thorecta reticulata*	Mero-sesquiterpenoid	Hunter Island Tasmania	Showed growth inhibition on SF-268, MCF-7, H460 and HT29 (GI_{50} = 5.1, 3.2, 5.1, and 10 μg/ml, respectively)	(Ovenden et al., 2011)
Dictyodendrin F (A34)	*Ianthella* sp.	Alkaloid	Bass Strait	Showed cytotoxicity against SW620 IC_{50} value of 8.5 μM	(Zhang et al., 2012)

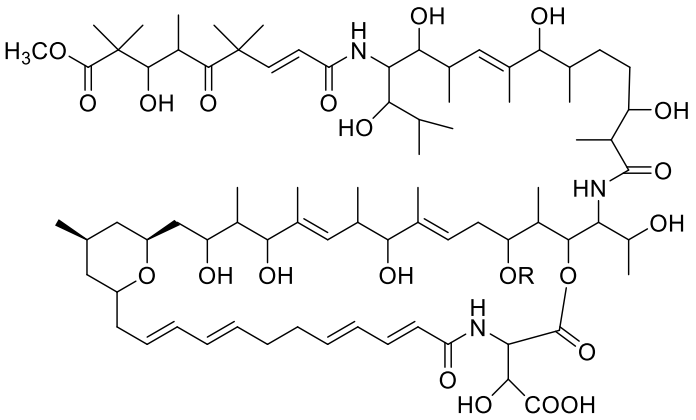
Dictyodendrin G (A35)	*Ianthella* sp.	Alkaloid	Bass Strait	Showed cytotoxicity against SW620 IC_{50} value of 2.0 μM	(Zhang et al., 2012)
Pesudoceralidinone A (A36)	*Pseudoceratina verrucosa*	Alkaloid	Hook Reef Lagoon	Showed cytotoxicity against HeLa and PC3, (IC_{50} = 44 and 35 μM, respectively)	(Tran et al., 2013)
Aplysamine 7 (A37)	*Pseudoceratina verrucosa*	Alkaloid	Hook Reef Lagoon	Showed cytotoxicity against HeLa and PC3, (IC_{50} = 19 and 4.9 μM, respectively)	(Tran et al., 2013)

Note: A549: human lung cancer cell line; HT29: human colon cancer cell line; P388: murine leukaemia; HM02: human stomach cancer cell line; HepG2: human liver cancer cell line; Huh7: human liver cancer cell line; MDA-MB-231: human breast cancer cell line; MM96L: human malignant melanoma (skin) cell line; MCF-7: human breast cancer cell line; A2780: human ovarian cancer cell line; DU145: human prostate cancer cell line; SF-268: human central nervous system-glioblastoma cell line; H460: human lung-large cancer cell line; SW620: human colon cancer cell line.

The chemical structural of these compounds (A1-37) are shown as below:



A 1



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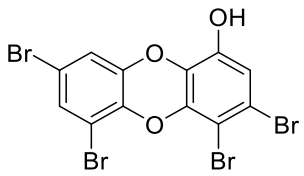

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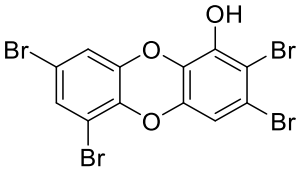

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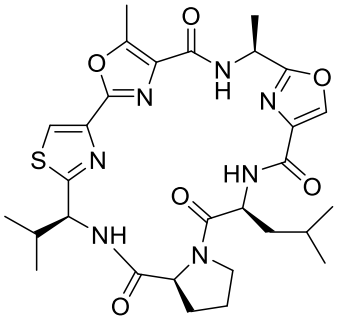

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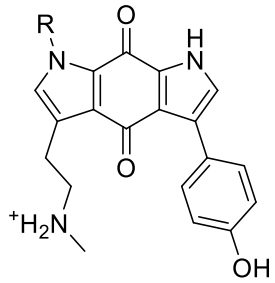
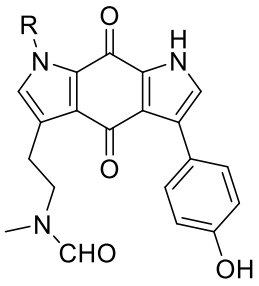
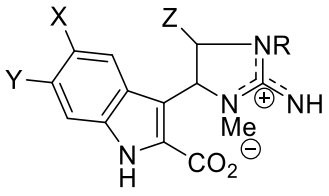


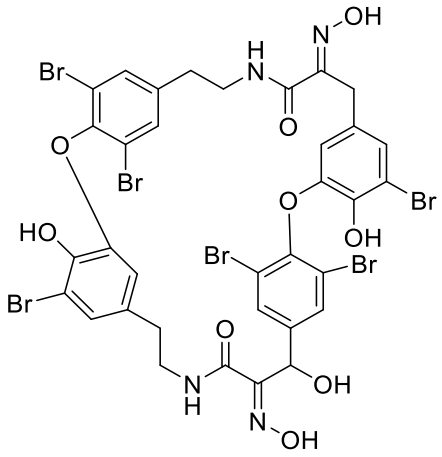
A 6 R = Me
A 7 R = H



A 8 R = Me
A 9 R = H



	X	Y	Z	R
A 10	Br	H	H	H
A 11	Br	H	H	Me
A 12	Br	OH	H	H
A 13	Br	OH	H	Me
A 14	Br	H	OH	Me
A 15	Br	OH	OH	Me



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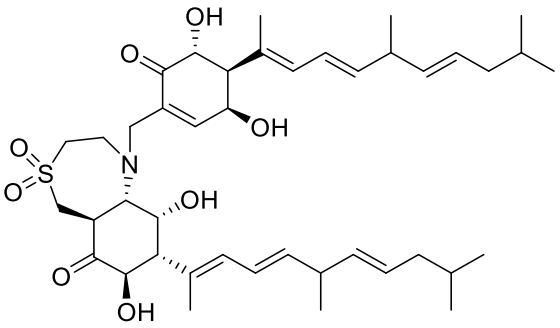

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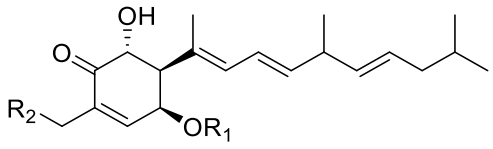
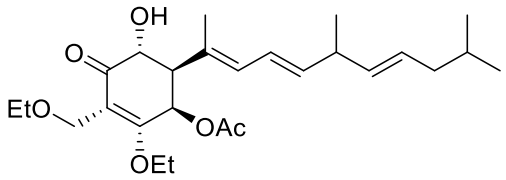
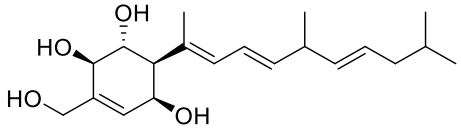


	R_1	R_2
A 18	H	H
A 19	Ac	H
A 20	Ac	OEt



A 21



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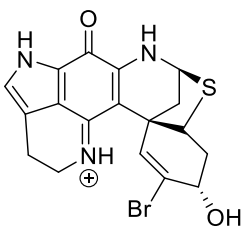

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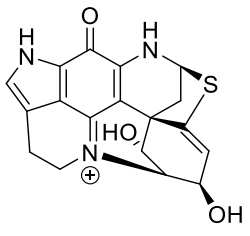

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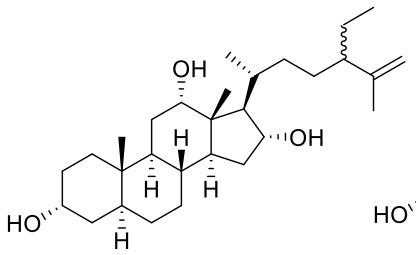
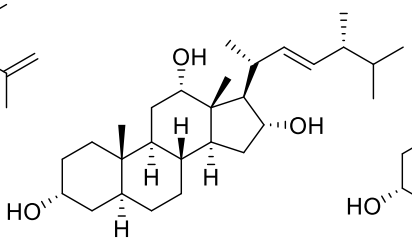


A 25



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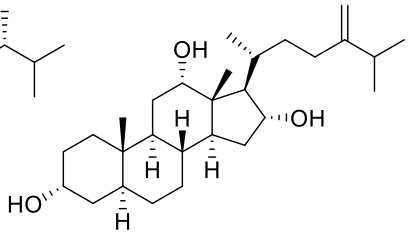
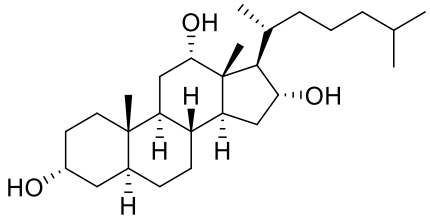


A 27



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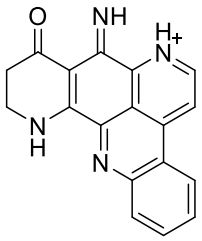

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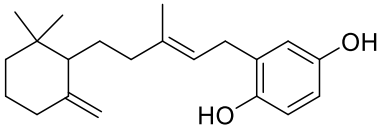

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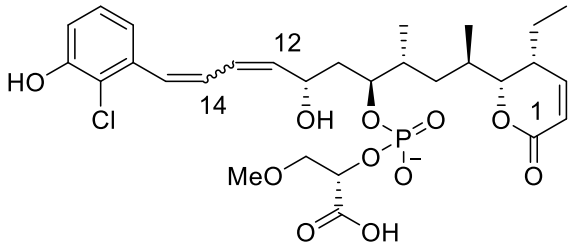
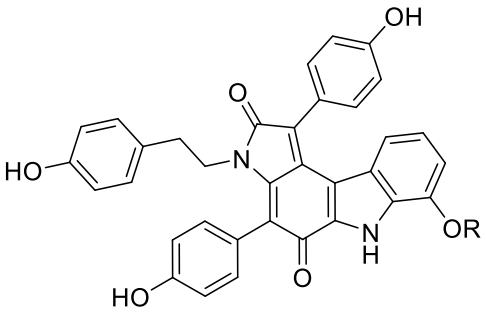
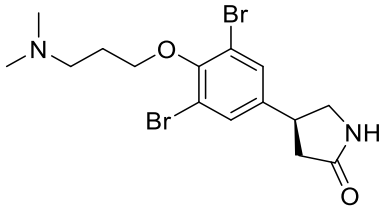


A 30 12Z, 14Z
A 31 12E, 14E
A 32 12E, 14Z



A 34 R = H
A 35 R = Me



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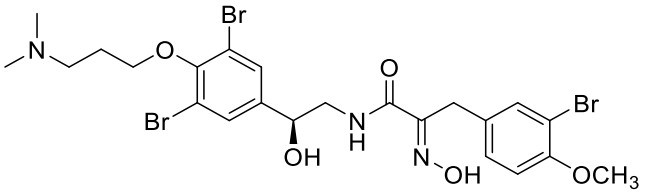


A 37

1.3The chemical constituents of marine sponges *Aplysilla*

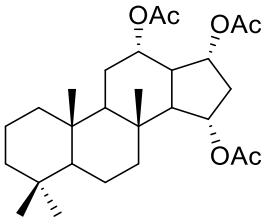
In this section, the literature review of the chemical constituents of the three marine sponges (*Aplysilla rosea*, *Chondropsis* sp. and *Tedania cf. anhelans*) mainly used in this study, particularly, genus *Aplysilla* will be reviewed. There are five different species that have been studied in sponge genus of *Aplysilla*, and a total of 56 compounds have been described for this genus. Among these compounds, diterpenes are the most common compounds discovered by far. These five species are *Aplysilla rosea*, *Aplysilla tango, Aplysilla sulphurea, Aplysilla polyrhaphis,* and *Aplysilla glacialis*.

1.3.1 *Aplysilla rosea*

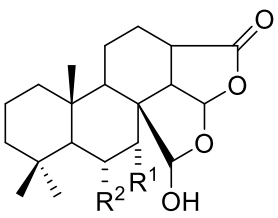
Sponge *Aplysilla rosea* (Class: Demospogiea, Order: Demospongiae, Family: Darwinellidae, genus: *Aplysilla*) (Figure 1.8) are thinly encrusting red/pink coloured sponges. It can be widely found ranging from Atlantic, North Sea, Sweden, Ireland, Channel and Mediterranean (Ackers et al., 1992).

Figure 1.8: Aplysilla rosea
Photo was taken by Dr Jason Tanner during sponges' collection in 2011.

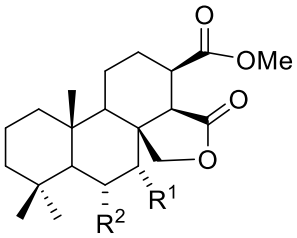
In 1979, the first novel compound (aplysillin) was obtained from New Zealand sponge *Aplysilla rosea* (Kazlauskas et al., 1979). Seven years later, Karuso and Taylor reported another eight compounds (aplyroseol -1 to -6 and two lactones AP1 and AP2) isolated from Australian sponge *Aplysilla rosea* (Karuso and Taylor, 1986). Later, 13 more diterpenes (aplyroseol -8 to -18, dendrillol-1 and -2) were reported (Taylor and Toth, 1997). Betancur-Glavis (2002) reported the growth inhibition activity of Dendrillol-1 against human cervix cancer cell line (HeLa), and larynx cancer cell line (Hep-2) with GI_{50} value of 13 μg/ml and 13 μg/ml, respectively.



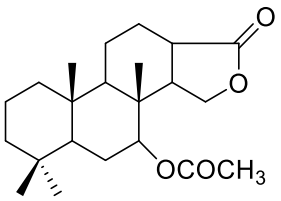
Aplysillin



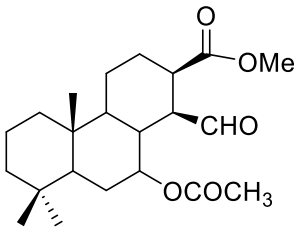
	R_1	R_2
Aplyroseol-1	$OCOC_3H_7$	H
Aplyroseol-2	$OCOCH_3$	OH
Aplyroseol-3	$OCOC_3H_7$	OH
Aplyroseol-4	$OCOC_3H_7$	$OCOCH_3$
Aplyroseol-5	OH	$OCOC_3H_7$
Aplyroseol-6	$OCOCH_3$	$OCOC_3H_7$



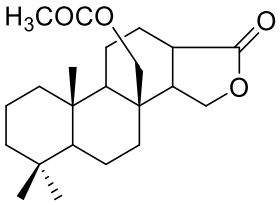
	R_1	R_2
Aplyroseol-8	$OCOC_3H_7$	H
Aplyroseol-9	$OCOCH_3$	H
Aplyroseol-10	OH	$OCOC_3H_7$
Aplyroseol-11	$OCOCH_4$	$OCOC_3H_7$
Aplyroseol-12	$OCOC_3H_7$	$OCOCH_3$



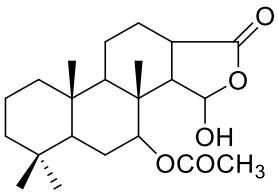
Aplyroseol-7



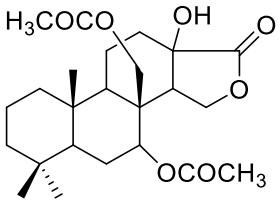
Aplyroseol-13



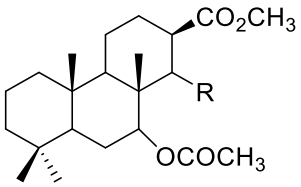
Aplyroseol-14



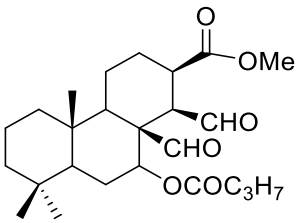
Aplyroseol-15



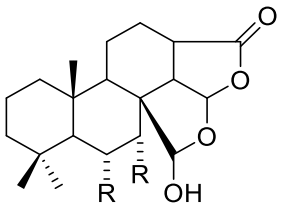
Aplyroseol-16



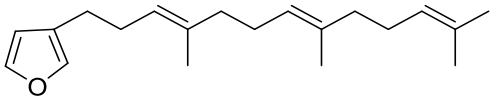
Aplyroseol-17 R = α-OH
Aplyroseol-18 R = β-OH



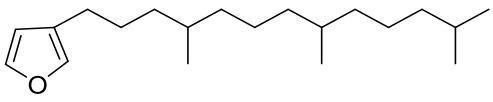
Aplyroseol-19



Dendrillol-1 R = H
Dendrillol-2 R = OAc



Lacton-AP1

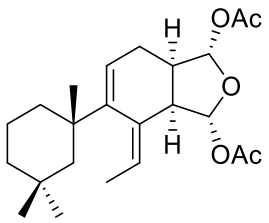


Lactone-AP2

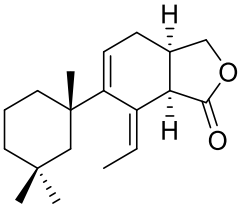
1.3.2 *Aplysilla tango*

Aplysilla tango (family Darwinelidae, order Dendroceratida) is an encrusting sponge with an orange colour. In 1990, five new diterpenes (gracilin A, aplytandiene-1, 2, and

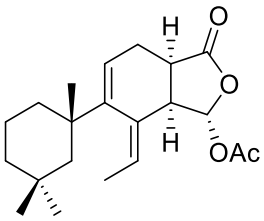
aplytangene-1, 2) together with seven steroids (steroids 1-7) were reported (Poiner and Taylor, 1990).



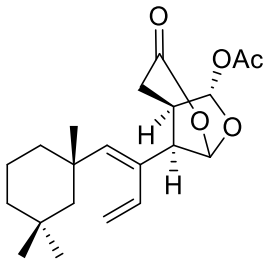
Gracilin A



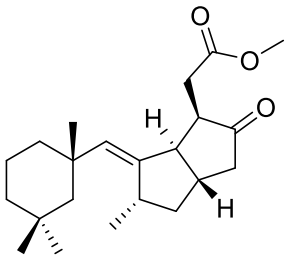
Aplytandiene-1



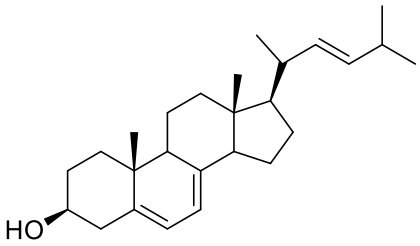
Aplytandiene-2



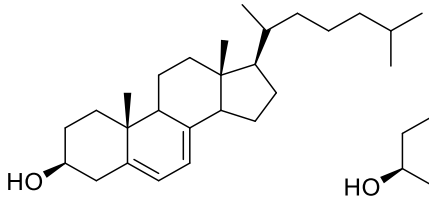
Aplytangene-1



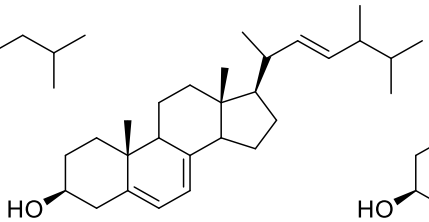
Aplytangene-2



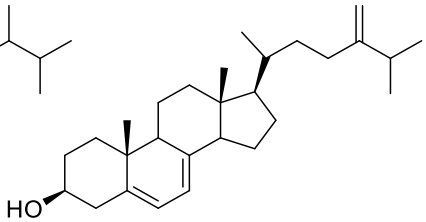
Steroid-1



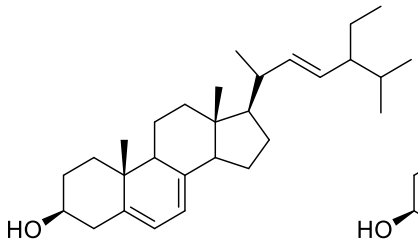
Steroid-2



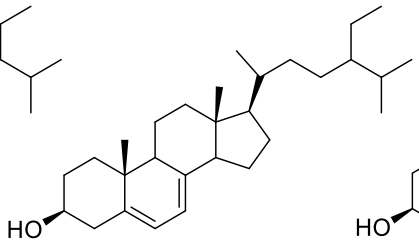
Steroid-3



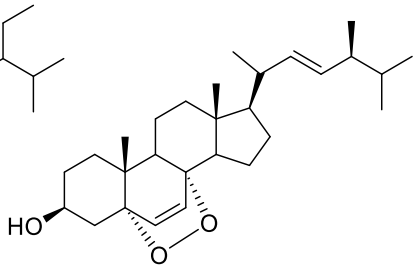
Steroid-4



Steroid-5



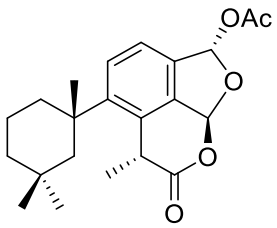
Steroid-6



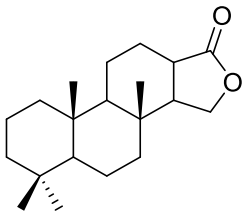
Steroid-7

1.3.3 *Aplysilla sulphurea*

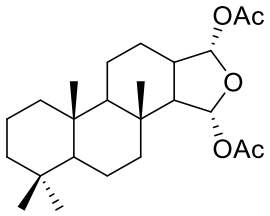
Aplysilla sulphurea (family Darwinelidae, order Dendroceratida) is an encrusting sponge with bright yellow colour. The first report on *Aplysilla sulphurea* (collected from Jervis Bay, Australia) was in 1984. A diterpene (aplysulphurin) was isolated from this sponge and determined the structure by single-crystal X-ray and spectrum data (Karuso et al., 1984b). Another two metabolites (16-oxospongian, and 15,16-diacetoxyspongian) were isolated from *Aplysilla sulphurea* 13 years later (Taylor and Toth, 1997). There is no bioactivity study on these compounds.



Aplysulphurin



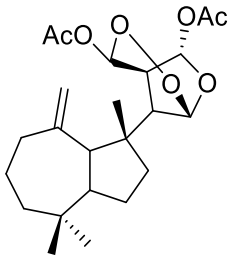
16-oxospongian



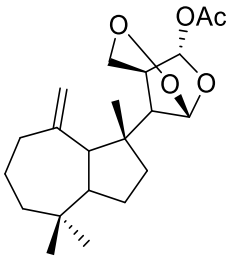
15,16-diacetoxyspongian

1.3.4 *Aplysilla polyrhaphis*

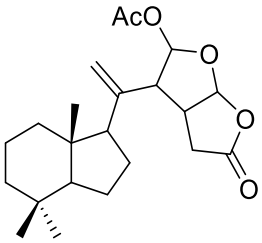
Aplysilla polyrhaphis (family Darwinelidae, order Dendroceratida) is an encrusting sponge with purple colour. In 1989, *Aplysilla polyrhaphis*, collected from the Gulf of California, U.S, contained four new diterpenes, polyrhaphins A-D, together with known compounds macfarlandin E, aplyviolene, norrisolide, shahamin, and γ-lactone (Bobzin and Faulkner, 1989). Chemical structures of these compounds are shown below:



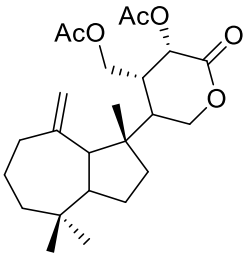
Macfarlandin E



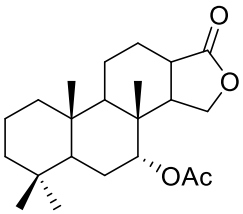
Aplyviolene



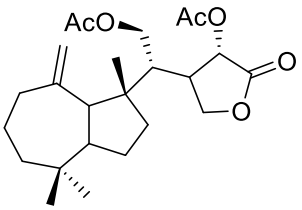
Norrisolide



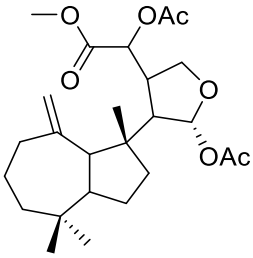
Shahamin



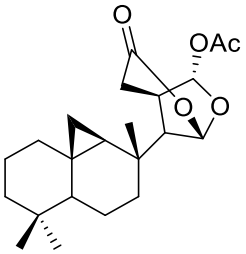
γ-lactone



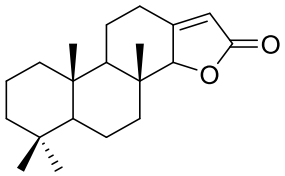
Polyrhaphins A



Polyrhaphins B



Polyrhaphins C

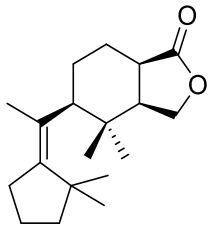


Polyrhaphins D

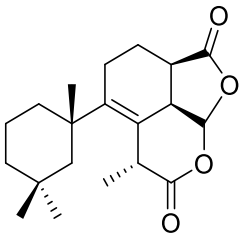
1.3.5 *Aplysilla glacialis*

Aplysilla glacialis (family Darwinelidae, order Dendroceratida) was first reported in 1989, and contained a novel diterpene with new carbon skeleton, Glaciolide (Tischler and Andersen, 1989). Two years later, the same group found four new terpenoids, cadlinolide A, B and aplysillolide A, B, and one novel diterpene, marginatone from sponge *Aplysilla glacialis* collected from Sadford Island (Barkley Sound, British Columbia.) (Tischler et al., 1991). Glaciasterols A and B were isolated from marine

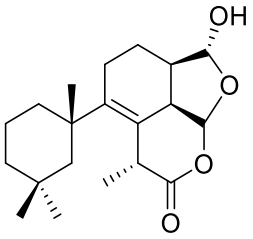
sponge *Aplysilla glacialis* (collected from Sydney Inlet and Barkley Sound, British Columbia) in 1992. Glaciasterol A showed cytotoxicity against murine leukemia L1210 and human breast cancer cell line (T47D) with ED_{50} value of 7 and 20 μg/ml, respectively (Pika et al., 1992).



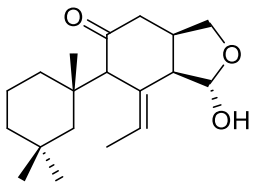
Glaciolide



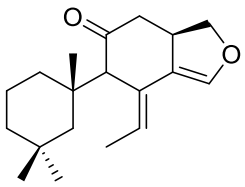
Cadlinolide A



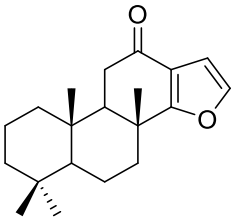
Cadlinolide B



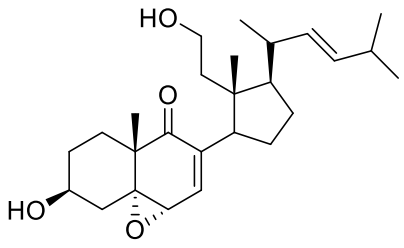
Aplysillolide A



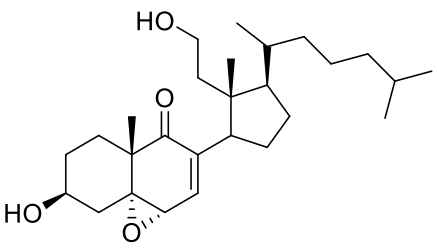
Aplysillolide B



Marginatone



Glaciasterol A



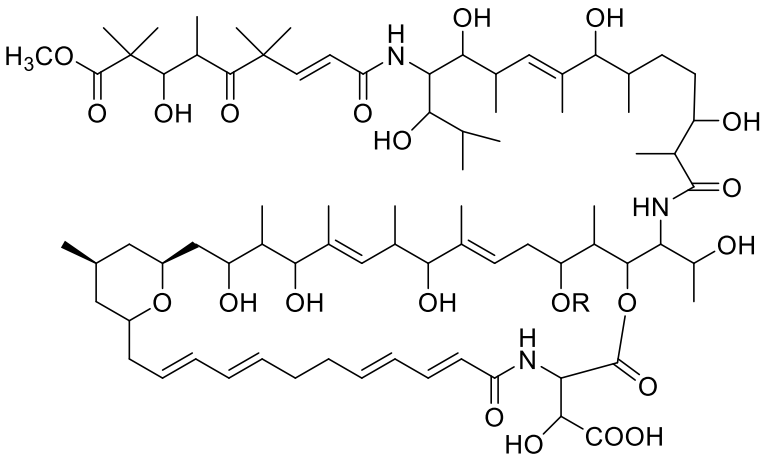
Glaciasterol B

1.3.6 *Chondropsis* sp.

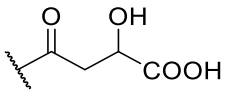
Sponge *Chondropsis* sp. (genus *Chondropsis*, Family Chondropsidae, Order Poecilosclerida) (Figure 1.9) is an upright, twisted ochre-orange walls sponge with tiny pores on the sides and oscula mounted flush with the surface of the ridges of the walls.

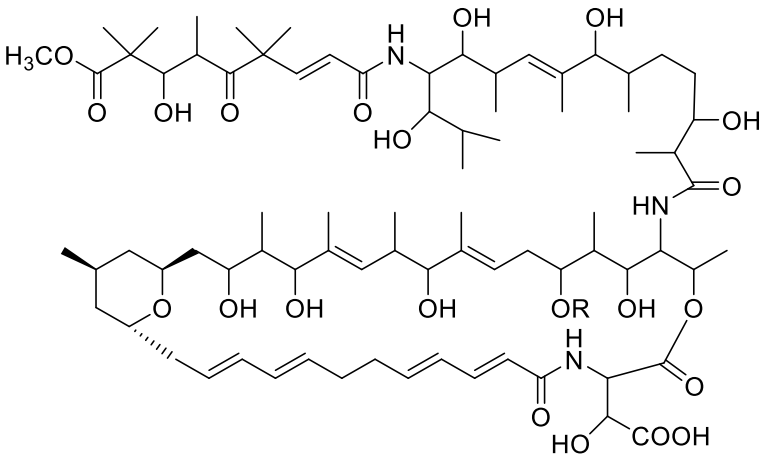
Figure 1.9: *Chondropsis* sp.
Photo was taken by Dr Jason Tanner during sponges' collection in 2011

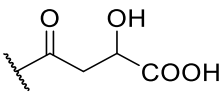
Two macrolide lactams, chondropsins A and B, isolated from an aqueous extract of sponge *chondropsis* sp. (Bass Island, Wollongong, Australia) was reported in 2000, and then another following study reported a macrolide lactam chondropsin D (Cantrell et al., 2000b, Rashid et al., 2001). The anti-cancer activity of chondropsin A was evaluated against 60 human tumour cell lines in the U.S. National Cancer Institute (NCI). The data showed this compound have potent, differential growth inhibition of the 60 cancer cell lines with a mean-panel GI_{50} value of 2.4×10^{-8} M and a range of $> 10^{3}$ in relative sensitivities of the individual cell lines (Cantrell et al., 2000b).



Chondropsin A R = H

Chondropsin B R = 



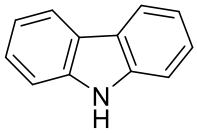
Chondropsin D R = 

1.3.7 *Tedania cf. anhelans*

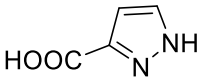
Sponge *Tedania cf. anhelans* (Family Tedaniidae, Order Poecilosclerida) (Figure 1.10) is a massive cushion shaped soft, reddish brown/yellow sponge with thin projections and elevated oscules. Surface smooth, consistency soft.

Figure 1.10: ***Tedania cf. anhelans***
Photo was taken by Dr Jason Tanner during the sponges' collection in 2011.

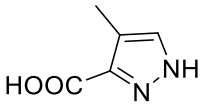
Two unusual heteroaromatic acids, pyrazole-3(5)-carboxylic acid and 4-methylpyrazole-3(5)-carboxylic acid isolated from sponge *Tedania cf. anhelans* (western Gujarat coast of India), were reported for the first time. The other compounds isolated were *p*-hydroxybenzaldehyde, phenylacetamide, 3-phenylpropionic acid, 3-(*p*-hydroxyphenyl)propionic acid, 3-(*p*-methoxyphenyl)propionic acid, β-carboline, and two diketopiperazines pro-val and pro-leu (Parameswaran et al., 1997a). There is no further bioactivity study on these compounds.



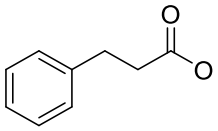
β-carboline



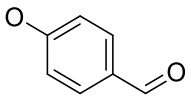
pyrazole-3(5)-carboxylic acid



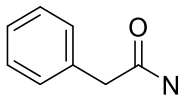
4-methylpyrazole-3(5)-carboxylic acid



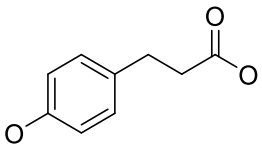
3-phenylpropionic acid



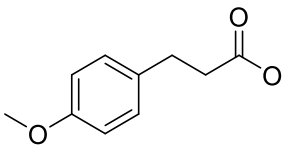
p-hydroxybenzaldehyde



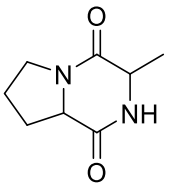
phenylacetamide



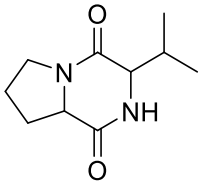
3-(*p*-hydroxyphenyl)propionic acid



3-(*p*-methoxyphenyl)propionic acid



diketopiperazine pro-leu



diketopiperazine pro-val

1.4Scope and aims

The overall aim of this study was to screen and characterize marine sponges from South Australian waters for discovering sponge derived compounds with potential anti-breast cancer properties. The outline of the scope of this study is presented in Figure 1.7.

To understand the anti-cancer activity of crude extracts from thirty South Australian marine sponges, the primary screening used three human breast cancer cell lines and a human breast non-cancer cell line as *in vitro* models: T47D (ductal carcinoma, an endogenously expressing mutant p53 cell line), MCF7 (adenocarcinoma, a p53 wild type), MDA-MB-231(adenocarcinoma, an endogenously expressing mutant p53 cell line), and 184B5 (human breast epithelial cell line). The potential cytotoxicity of the three crude extracts from *Aplysilla rosea*, *Chondropsis* sp. and *Tedania cf. anhelans* were determined via crystal violet assay. Furthermore, cell cycle progression and apoptosis induction were measured by propidium iodide (PI) staining and PI and Annexin V-FITC (fluorescein isothiocyanate) staining and the endpoint detected by flow cytometry. Sponge *Aplysilla rosea* was chosen for further study because it showed both cell cycle and apoptosis induction against human breast cancer cell lines and with selective cytotoxicity against breast cancer cell lines over normal breast cell line (see Chapter two).

Sponge *Aplysilla rosea* were recollected as the same location as the first time screening study. Over 50 compounds were isolated and purified from *Aplysilla rosea* by using column chromatography, and preparative HPLC. Four novel compounds and 20 known compounds were unambiguously elucidated by spectroscopic analyses, as well as by

comparison with the literature data. The absolute configuration of one of the new compounds was determined by using X-ray diffraction analysis (see Chapter three).

The cytotoxicity evaluation revealed that 12 pure compounds showed higher potent cytotoxicity against human breast cancer cell lines (T47D, MCF-7 and MDA-MB-231) by using the crystal violet assay. Especially, compound **7** indicated selective cytotoxicity against breast cancer cell lines over normal breast cell line (184B5). A further cell cycle and apoptosis induction assays were carried out on compound **7**, and it showed great potential anti-breast cancer properties (see Chapter four).

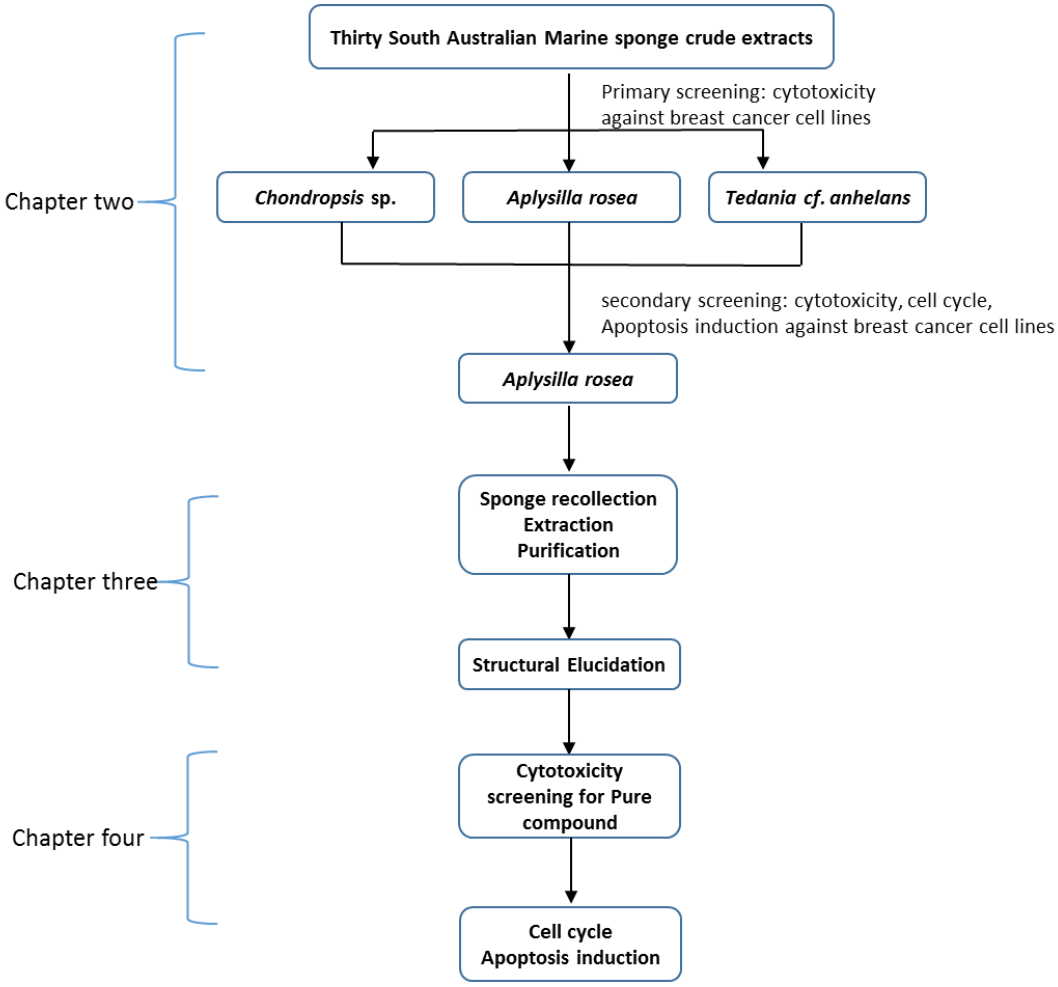


Figure 1.7: Brief outline of scope of thesis for anti-breast cancer properties and constituents of South Australian marine sponge *Aplysilla rosea*

Chapter 2: Cytotoxicity against Breast Cancer Cells of South Australian Marine Sponge Extracts

2.1 Introduction

Marine sponges [Porifera] are the single best sources of novel marine natural products, accounting for over 30% of all marine natural products discovered by far. Furthermore and marine sponges are a rich source of bioactive marine natural products, especially with nearly 50% exhibiting potential anticancer activities (Munro et al., 1999, Molinski et al., 2009, Gerwick and Moore, 2012). The two derivatives, Cytarabine and Eribulin Mesylated from natural products of marine sponges have been approved by the United States Food and Drug Administration for cancer treatment (Simmons et al., 2005, Gerwick and Moore, 2012).

Oceans in Southern Australia have largely untapped resource of marine sponges with an estimated number of over 800 species, and approximately 58% are endemic species in Australia (Hooper and Lévi, 1994). Therefore, the aim of this study presented in this chapter is to screen anti-cancer properties of the crude extracts obtained from untapped marine sponges from South Australian waters on human breast cancer cell lines (T47D, MCF7 and MDA-MB-231). Cell viability was assessed by crystal violet assay, and cell cycle progression using PI staining was investigated by flow cytometry. The apoptotic effect of the crude extracts was investigated using Annexin V and PI staining.

The specific objectives of this chapter are to:

1. Evaluate the cytotoxicity of the crude extracts from 30 South Australian marine sponges against three breast cancer cell lines and identify the sponge extracts with significant bioactivities for further study.

2. Evaluate the cytotoxicity (time and dose dependent), cell cycle arrest and apoptosis induction of selected sponge extracts against three breast cancer cell lines.

2.2 Materials and Methods

2.2.1 Animal Material

Thirty sponges were collected around Outer Harbor and Rapid Bay of South Australian in 2011 and 2012 by Dr Jason Tanner and his team (South Australia Research and Development Institute, Australia), which were identified by Ms Shirley Sorokin (Centre for Marine Bioproducts Development, Flinders University, Australia). Thirty voucher samples (No. 051211.01-19, 160512.01-11) were deposited in the Centre for Marine Bioproducts Development, Flinders University, Australia.

2.2.2 Extraction and sample preparation

Thirty fresh sponges (50-200g, wet weight) were freeze-dried (VirTis, BenchTop K, USA). The dried specimens were extracted with methanol and chloroform (200ml 1:1 v/v, Merck, Germany) at room temperature for four times (Ebada et al., 2008a, Sikorska et al., 2012). Crude extracts were concentrated under reduced pressure at 30 °C using a rotary evaporator (EYELA, Japan) to remove organic solvents. Crude extracts were redissolved in DMSO at 10 mg/ml and stored at -20 °C.

2.2.3 Cell lines and cell culture maintenance

Three human breast cancer cell lines MCF7 (ATCC Number HTB-22), MDA-MB-231 (ATCC Number HTB-26) and T47D (ATCC Number HTB-133) and human normal breast cell line 184B5 were obtained from the American Type Culture Collection. The details of these cell lines are listed in Table 2.1.

Table 2.1: Summary of the cell lines used in this study

Cell line	ATCC number	Origin and cell type	Receptors	Disease	Age/gender of donor
T47D	HTB-133	Breast epithelium	ER+, PR+	Ductal carcinoma	54/F
MCF-7	HTB-22	Breast epithelium	ER+, PR+	Adenocarcinoma	69/F
MDA-MB-231	HTB-26	Breast epithelium	ER-, PR-, HER2+	Adenocarcinoma	51/F
184B5	CRL-8799	Breast epithelium	N/A	Normal	21/F

Cells were cultured in DMEM medium (for MCF7 and MDA-MB-231), RPMI medium (for T47D) (sigma, USA) or MEBM medium (for 184B5) (Lonza, VIC, Australia) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Invitrogen Corporation, Australia), 1% penicillin and 0.1 mg/ml streptomycin (Thermo Scientific, Melbourne, Australia). Cells were maintained in a fully humidified incubator with 5% CO_2 at 37 °C.

Cell lines were grown to 80-90% confluence in T25cm^2 or T75cm^2 cell culture flask (Nunc, Roskilde, Denmark) in an incubator. Cells were washed with phosphate-buffered saline (PBS; see Appendix A1) and detached with 0.5 ml or 1 ml 0.25% trypsin-EDTA (Thermo Scientific, Melbourne, Australia). Fresh medium (2 ml or 5 ml) was added into the flask. The medium

was removed after cell suspension centrifuged at 1200 rpm for 5 min, and the cell pellet was resuspended with fresh medium.

2.2.4 Trypan Blue exclusion assay

The Trypan Blue exclusion assay can clearly differentiate between live and dead cells under the microscope. Because the Trypan Blue can stain dead and dying cells to blue as their cell membrane integrity is lost. In contrast, live cell membrane will exclude the penetrating of Trypan Blue and remains yellow in colour (Petty et al., 1995). In this thesis, the Trypan Blue exclusion assay was used to evaluate the cell concentration by cell counting. Briefly, 50 μl Trypan Blue (0.2%, w/v, Appendix A1) was added into 50 μl aliquots of cell suspension. The 20 μl resuspension aliquots were loaded on a haemocytometer for counting. The concentration of viable cell and the viability % were calculated by the following formulas:

Concentration of viable cell (cells/ml)

= the number of live cells $\times 2 \times 10^4$/ the number of squares

Viability (%)

= the number of live cells / (the number of live cells + the number of dead cells) $\times$ 100%

The cultured cells with a viability over 90% were used in the experiments.

2.2.5 Cell viability test

Cell viability was measured using the crystal violet assay (Saotome et al., 1989, Ishiyama et al., 1996). Briefly, cells were seeded in 96-well flat bottom plates at 1×10^4 cells/well with 100 μl media and incubated for 24 h. Cells were then treated with 100 μl media (use as untreated control), 100 μl 0.5% DMSO in media (use as vehicle control) and 100 μl varying

concentrations of sponge crude extracts in DMSO for the required time (initially 48 h, then time dependent test using 24, 48 and 72 h). After treatment, media and dead cells were washed away, and the remaining cells were stained with 0.5% (w/v) crystal violet and de-stained with 33% acetic acid. OD was measured on a microplate reader (Bio-Tek Instruments Inc, USA) at 570 nm (Saotome et al., 1989) and the values were converted to cells/well based on a standard curve (625 to 40000 cells/well) with each experiment. The effect of sponge extracts on cell viability was calculated by converting cells/well to percentage of cell survival compared to the untreated cells (%).

2.2.6 Cell cycle assay

Cell cycle distribution was evaluated by flow cytometry (Nicoletti et al., 1991). Cells were treated with varying concentration of sponge crude extracts in culture media for 48 h. After treatment, the cells were harvested by trypsinisation and then fixed in ice-cold 70% (v/v) ethanol at –20 °C overnight. Following the cell fixation, the fixed solution was removed and cells were resuspended in 1 ml of mixture buffer solution (20 μl/ml of PI and 200 μg/ml of RNase in 0.1% Triton X-100 in phosphate buffered saline (PBS)) and incubated in the dark for 30 min at room temperature (RT). The samples (approximate 20000 cells for each experiment) were analysis by Accuri C6 flow cytometry.

The respective medium is used to store cell in the liquid nitrogen, which containing 10% (v/v) FBS and 5% (v/v) dimethyl sulfoxide (DMSO). Cell lines store in the liquid nitrogen at concentration of 4×10^6 cells/ml.

2.2.7 Apoptosis analysis

Apoptosis analysis was done with Annexin V-FITC Apoptosis Detection kit (BD Biosciences, USA). Briefly, Cells were treated as described in cell cycle assessment, and then treated cells were washed with ice-cold 0.1% (w/v) sodium azide in PBS for two times. The cell pellets were resuspended in 1 × binding buffer at 10^6 cells/ml. A 100 ml testing sample was transferred into a culture tube, and stained with kit mixture (5 μl of Annexin V-FITC and 5 μl of PI) at RT for 15 min. After staining, testing samples were resuspended with 400 μl of binding buffer, and then analysed by Accuri C6 flow cytometry to evaluate apoptosis frequency. A total of 20,000 cells were analysed for each assessment.

2.2.8 Statistical analysis

All the experiments were replicated at least three independent times and data are presented as mean ± S.E.M. The IC_{50} value was calculated by using GraphPad prism V. 6.01 for Windows software. One way ANOVA was used to analyse the data. Tukey's HSD *post hoc* (equal variances) was used in the subsequent analysis. Statistics tests were performed using IBM SPSS Statistics 22 software. Significant difference was considered as when the *P*-value was less than 0.05.

2.3 Results

2.3.1 Sponge Identification

The sponge samples were collected from two different locations, Rapid Bay (December, 2011) and Outer Harbor (May, 2012) in South Australia. The 30 sponge samples used in this study belong to 10 different orders in the class of Demospongiae. 13 sponge samples belong to the order Poecilosclerida, six sponge samples belong to order Dictyoceratida, three sponge samples

belong to Tetractinellida, two sponge samples belong to Haplosclerida, and other 6 sponge samples belong to order Verongiida, Dendroceratida, Astrophoria, Clionaida, Leucosolenida and Tethyida, respectively. The details of taxonomic identifications of these sponge samples are reported in Table 2.2.

Table.2.2: Sponge identifications

Collection Code	Sponge sample	Genus	Family	Order	Collected Location
051211.01	*Suberea* sp.	*Suberea*	Aplysinellidae	Verongiida	Rapid Bay
051211.02	*Psammocinia* sp.1	*Psammocinia*	Irciniidae	Dictyoceratida	Rapid Bay
051211.03	*Ecionemia* sp.1	*Ecionemia*	Ancorinidae	Tetractinellida	Rapid Bay
051211.04	Geodiidae sp.	/	Geodiidae	Tetractinellida	Rapid Bay
051211.05	*Ircinia* sp.	*Ircinia*	Irciniidae	Dictyoceratida	Rapid Bay
051211.06	*Tedania cf. anhelans*	*Tedania*	Tedaniidae	Poecilosclerida	Rapid Bay
051211.07	*Hyatella* sp.	*Hyatella*	Spongiidae	Dictyoceratida	Rapid Bay
051211.08	*Chondropsis* sp. 1	*Chondropsis*	Chondropsidae	Poecilosclerida	Rapid Bay
051211.09	*Clathria* sp. 1	*Clathria*	Microcioninae	Poecilosclerida	Rapid Bay
051211.10	*Aplysilla rosea*	*Aplysilla*	Darwinellidae	Dendroceratida	Rapid Bay

Table.2.2: Sponge identifications (continued)

Collection Code	Sponge sample	Genus	Family	Order	Collected Location
051211.11	*Psammocinia* sp.1	*Psammocinia*	Irciniidae	Dictyoceratida	Rapid Bay
051211.12	*Ecionemia* sp. 2	*Ecionemia*	Ancorinidae	Tetractinellida	Rapid Bay
051211.13	*Chondropsis cf. arenifera*	*Chondropsis*	Chondropsidae	Poecilosclerida	Rapid Bay
051211.14	*Geodia* sp.	*Geodia*	Geodiidae	Astrophoria	Rapid Bay
051211.15	*Thorectandra* sp.	*Thorectandra*	Trorectidae	Dictyoceratida	Rapid Bay
051211.16	*Spheciospongia* sp.	*Spheciospongia*	Clionaidae	Clionaida	Rapid Bay
051211.17	Esperiopsidae	/	Esperiopsidae	Poecilosclerida	Rapid Bay
051211.18	*Psammoclema* sp.	*Psammoclema*	Chondropsidae	Poecilosclerida	Rapid Bay
051211.19	*Sycon* sp.	*Sycon*	Sycettidae	Leucosolenida	Rapid Bay
160512.01	*Callyspongia* sp.	*Callyspongia*	Callyspongiidae	Haplosclerida	Outer Harbor

Table.2.2: Sponge identifications (continued)

Collection Code	Sponge sample	Genus	Family	Order	Collected Location
160512.02	*Euryspongia* sp. 1	*Euryspongia*	Dysideidae	Dictyoceratida	Outer Harbor
160512.03	*Strongylacidon* sp.1	*Strongylacidon*	Chondropsidae	Poecilosclerida	Outer Harbor
160512.04	*Clathria* sp. 2	*Clathria*	Microcioninae	Poecilosclerida	Outer Harbor
160512.05	*Chalinula* sp.	*Chalinula*	Chalinidae	Haplosclerida	Outer Harbor
160512.06	*Chondropsis* sp. 2	*Chondropsis*	Chondropsidae	Poecilosclerida	Outer Harbor
160512.07	*Crella* sp.	*Crella*	Crellidae	Poecilosclerida	Outer Harbor
160512.08	*Chondropsis* sp. 3	*Chondropsis*	Chondropsidae	Poecilosclerida	Outer Harbor
160512.09	*Strongylacidon* sp.2	*Strongylacidon*	Chondropsidae	Poecilosclerida	Outer Harbor
160512.10	*Tethya* sp.	*Tethya*	Tethyidae	Tethyida	Outer Harbor
160512.11	*Chondropsis* sp. 4	*Chondropsis*	Chondropsidae	Poecilosclerida	Outer Harbor

Sponges were collected from South Australian coastal waters during 2011 and 2012; codes refer to date (day month year. code) of collection. Taxonomic specimens of the sponges are kept at the Centre for Marine Bioproducts Development, Flinders University.

2.3.2 Primary screening

2.3.2.1 Cell viability assay on T47D cell line

In primary screening, the induction of cell killing was determined by crystal violet assay (relative cell number). Extracts from thirty sponge samples were tested on T47D human breast cancer cell line. The crystal violet assay indicated significant cytotoxicity ($P < 0.05$) after 48 h treatment with eleven sponge extracts from *Tedania cf. anhelans*, *Chondropsis* sp. 1, *Clathria* sp. 1, *Aplysilla rosea*, *Psammocinia* sp. 2, *Callyspongia* sp., *Strongylacidon* sp. 1, *Chalinula* sp., *Crella* sp., *Strongylacidon* sp. 2, and *Chondropsis* sp.4, at 50 μg/ml. Especially, three organic extracts, from sponges *Tedania cf. anhelans*, *Chondropsis* sp. 1 and *Aplysilla rosea*., inhibited cell growth greater than 90% at the dose of 50 μg/ml (Table 2.3).

Table 2.3: Viability assessed by relative cell number (%) of T47D cells estimated by crystal violet assay

Sponge	Viability assessed by relative cell number (%)		
	0.5 μg/ml	5 μg/ml	50 μg/ml
Suberea sp.	89.20 ± 6.82	99.72 ± 7.47	65.09 ± 7.63
Psammocinia sp.1	101.51 ± 7.93	118.98 ± 8.10	58.36 ± 1.07
Ecionemia sp.1	91.44 ± 5.23	104.52 ± 11.94	95.75 ± 6.95
Geodiidae sp.	98.85 ± 4.28	101.70 ± 12.67	114.72 ± 8.36
Ircinia sp.	82.72 ± 7.63	82.64 ± 5.33	65.26 ± 9.98
Tedania cf. anhelans	88.10 ± 7.51	58.37 ± 8.98	2.12 ± 1.58 *
Hyatella sp.	98.18 ± 4.38	105.94 ± 11.61	90.91 ± 6.02
Chondropsis sp. 1	85.32 ± 9.68	50.32 ± 11.47	2.56 ± 0.60 *
Clathria sp. 1	99.46 ± 5.04	100.19 ± 11.29	44.43 ± 4.69 *
Aplysilla rosea	89.20 ± 9.26	74.48 ± 4.09	7.60 ± 1.36 *
Psammocinia sp. 2	90.97 ± 9.01	105.20 ± 7.66	36.75 ± 10.28 *
Ecionemia sp. 2	102.96 ± 6.25	96.92 ± 2.75	91.24 ± 9.72
Chondropsis cf. arenifera	88.51 ± 4.66	81.07 ± 11.56	50.51 ± 6.19
Geodia sp.	96.41 ± 5.28	96.84 ± 7.72	94.08 ± 10.32
Thorectandra sp.	107.94 ± 9.96	113.22 ± 4.41	102.98 ± 7.84
Spheciospongia sp.	92.59 ± 3.24	98.97 ± 3.14	94.79 ± 6.45
Esperiopsidae	100.45 ± 6.79	92.65 ± 1.91	87.75 ± 7.64
psammoclema sp.	98.99 ± 2.99	97.79 ± 6.66	84.44 ± 7.11
Sycon sp.	90.71 ± 1.39	86.63 ± 6.43	83.64 ± 6.97
Callyspongia sp.	97.34 ± 3.56	77.72 ± 2.32	15.23 ± 2.90 *
Euryspongia sp.	97.15 ± 8.88	94.66 ± 7.37	96.51 ± 9.24
Strongylacidon sp. 1	113.13 ± 9.82	89.41 ± 6.18	40.14 ± 2.42 *
Clathria sp. 2	102.00 ± 9.50	98.47 ± 3.90	54.39 ± 5.28
Chalinula sp.	94.13 ± 5.18	54.17 ± 5.29	12.81 ± 2.18 *
Chondropsis sp. 2	102.00 ± 9.50	98.47 ± 3.90	54.39 ± 5.28
Crella sp.	103.14 ± 6.08	57.71 ± 5.33	38.37 ± 4.92 *
Chondropsis sp. 3	98.53 ± 10.37	96.07 ± 7.15	70.62 ± 4.57
Strongylacidon sp. 2	104.57 ± 10.73	73.06 ± 2.59	34.71 ± 3.32 *
Tethya sp.	105.02 ± 8.21	105.34 ± 6.45	102.91 ± 10.22
Chondropsis sp.4	102.49 ± 9.24	73.66 ± 4.89	14.96 ± 1.54 *

Note: In 96-well plates for 48 h exposure to 30 marine sponge crude extracts (at dose of 0.5, 5, 50 μg/ml). Data are shown as relative surviving cell numbers percentage compare to the vehicle control (untreated cells, 0 μg/ml) and are mean of three replicates ± S.E.M. *Significantly different from vehicle control (0 μg/ml) $P < 0.05$.

2.3.2.2 Cell viability assay on MCF7 cell line

Organic extracts of all thirty sponges were tested by crystal violet assay on MCF7 breast cancer cell line. Among these crude extracts, *Tedania cf. anhelans*, *Chondropsis* sp. 1, *Clathria* sp. 1, *Aplysilla rosea*, *Psammocinia* sp. 2, *Chondropsis cf. arenifera*, *Strongylacidon* sp. 1, *Chalinula* sp., *Chondropsis* sp. 2, *Chondropsis* sp. 3, *Tethya* sp., and *Chondropsis* sp. 4 showed the most significant ($P < 0.05$) cytotoxicity against MCF7 cells at 50 μg/ml. Especially, organic extracts, from sponges *Tedania cf. anhelans*, *Chondropsis* sp. 1, *Aplysilla rosea*, *Chalinula* sp., *Chondropsis* sp. 3 inhibited more than 90% cell growth at the dose of 50 μg/ml (Table 2.4).

Table 2.4: Viability assessed relative cell number (%) of MCF7 cells estimated by crystal violet assay

Sponge code	Viability assessed by relative cell number (%)		
	0.5 μg/ml	5 μg/ml	50 μg/ml
Suberea sp.	90.62 ± 8.40	79.72 ± 5.30	55.30 ± 10.67
Psammocinia sp.1	98.86 ± 13.28	91.31 ± 6.44	63.49 ± 4.55
Ecionemia sp.1	79.34 ± 6.07	86.09 ± 5.21	84.39 ± 6.62
Geodiidae sp.	99.82 ± 4.25	96.93 ± 11.71	102.60 ± 3.95
Ircinia sp.	79.94 ± 11.85	83.76 ± 5.73	72.95 ± 3.23
Tedania cf. anhelans	77.48 ± 10.18	74.22 ± 9.93	8.42 ± 5.98 *
Hyatella sp.	85.61 ± 7.27	94.45 ± 5.89	72.98 ± 1.87
Chondropsis sp. 1	72.74 ± 11.12	55.32 ± 4.05	6.17 ± 3.72 *
Clathria sp. 1	98.05 ± 5.01	94.01 ± 9.43	49.47 ± 6.92 *
Aplysilla rosea	84.40 ± 7.99	78.40 ± 2.20	9.60 ± 3.67 *
Psammocinia sp. 2	102.64 ± 9.34	108.15 ± 3.64	46.95 ± 4.50 *
Ecionemia sp. 2	108.66 ± 1.86	112.93 ± 8.52	99.34 ± 11.70
Chondropsis cf. arenifera	106.05 ± 10.06	100.28 ± 8.41	48.99 ± 6.82 *
Geodia sp.	104.74 ± 5.20	94.42 ± 8.11	93.45 ± 9.54
Thorectandra sp.	98.24 ± 2.82	100.37 ± 9.53	96.55 ± 6.49
Spheciospongia sp.	91.51 ± 3.79	101.76 ± 7.94	102.31 ± 8.36
Esperiopsidae	109.64 ± 5.73	100.58 ± 6.14	102.89 ± 3.19
psammoclema sp.	97.84 ± 2.81	104.10 ± 9.62	95.15 ± 7.03
Sycon sp.	100.35 ± 7.03	104.46 ± 3.97	91.69 ± 8.13
Callyspongia sp.	110.81 ± 7.34	114.08 ± 9.66	73.94 ± 7.82
Euryspongia sp.	93.43 ± 10.60	97.33 ± 2.90	83.62 ± 3.43
Strongylacidon sp. 1	90.99 ± 2.74	98.44 ± 1.75	42.98 ± 4.55 *
Clathria sp. 2	99.32 ± 6.94	89.82 ± 3.06	61.05 ± 0.32
Chalinula sp.	67.11 ± 9.20	56.74 ± 4.89	6.40 ± 2.19 *
Chondropsis sp. 2	112.13 ± 4.68	61.32 ± 0.98	34.29 ± 5.44 *
Crella sp.	60.67 ± 1.97	67.94 ± 4.95	51.94 ± 1.44
Chondropsis sp. 3	70.55 ± 7.05	63.20 ± 2.46	9.50 ± 4.44 *
Strongylacidon sp. 2	115.03 ± 7.56	116.53 ± 8.40	116.36 ± 1.47
Tethya sp.	112.82 ± 4.34	59.00 ± 3.56	14.47 ± 3.22 *
Chondropsis sp.4	113.12 ± 5.21	92.96 ± 7.70	18.54 ± 7.33 *

Note: In 96-well plates for 48 h exposure to 30 marine sponge crude extracts (at dose of 0.5, 5, 50 μg/ml). Data are shown as relative surviving cell numbers percentage compare to the vehicle control (untreated cells, 0 μg/ml) and are mean of three replicates ± S.E.M. *Significantly different from vehicle control (0 μg/ml) $P < 0.05$.

2.3.2.3 Cell viability assay on MDA-MB-231 cell line

Thirty marine sponge crude extracts were tested by the crystal violet assay (relative cell number) on MDA-MB-231 breast cancer cell line. In the crystal violet assay, fourteen crude extracts (*Tedania cf. anhelans*, *Chondropsis* sp. 1, *Clathria* sp. 1, *Aplysilla rosea*, *Psammocinia* sp. 2, *Chondropsis cf. arenifera*, *Callyspongia* sp., *Strongylacidon* sp. 1, *Clathria* sp. 2, *Chalinula* sp., *Chondropsis* sp. 2, *Chondropsis* sp. 3, *Tethya* sp., and *Chondropsis* sp. 4) presented a significant ($P < 0.05$) cytotoxicity against MDA-MB-231 cells at 50 μg/ml. Three extracts from *Tedania cf. anhelans*, *Chondropsis* sp. 1 and *Aplysilla rosea* inhibited cell growth by 90% at the dose of 50 μg/ml (Table 2.5).

2.3.2.4 Summary from primary screening

In primary screening, eight crude extracts (*Tedania cf. anhelans*, *Chondropsis* sp. 1, *Clathria* sp. 1, *Aplysilla rosea*, *Psammocinia* sp. 2, *Strongylacidon* sp. 1, *Chalinula* sp., and *Chondropsis* sp. 4) exhibited significant cytotoxicity against all three breast cancer cell lines at a dose of 50 μg/ml. Only the three extracts from sponge *Tedania cf. anhelans*, *Chondropsis* sp. 1 and *Aplysilla rosea* inhibited cell growth more than 90% at the dose of 50 μg/ml. Therefore, these three sponge extracts were chosen for further study.

Table 2.5: Viability assessed by relative cell number (%) of MDA-MB-231 cells estimated by crystal violet assay

Sponge code	Viability assessed by relative cell number (%)		
	0.5 μg/ml	5 μg/ml	50 μg/ml
Suberea sp.	99.14 ± 11.21	87.00 ± 4.33	60.24 ± 9.14
Psammocinia sp.1	88.62 ± 10.58	83.17 ± 8.51	73.41 ± 2.08
Ecionemia sp.1	91.20 ± 11.91	102.06 ± 8.02	91.29 ± 0.63
Geodiidae sp.	98.10 ± 3.48	95.70 ± 2.15	101.90 ± 8.43
Ircinia sp.	146.97 ±7.01	108.20 ± 3.19	87.31 ± 6.73
Tedania cf. anhelans	90.20 ± 7.89	77.52 ± 7.73	2.60 ± 1.94 *
Hyatella sp.	80.50 ± 9.73	84.33 ± 3.94	63.32 ± 9.67
Chondropsis sp. 1	84.30 ± 7.15	43.37 ± 9.72 *	5.24 ± 0.71 *
Clathria sp. 1	100.11 ± 8.02	100.60 ± 8.01	48.87 ± 8.61 *
Aplysilla rosea	84.54 ± 6.74	86.00 ± 1.82	6.24 ± 0.70 *
Psammocinia sp. 2	114.43 ± 5.03	114.68 ± 7.73	11.71 ± 5.91 *
Ecionemia sp. 2	113.00 ± 7.77	94.45 ± 4.08	94.94 ± 11.19
Chondropsis cf. arenifera	89.13 ± 8.77	51.79 ± 7.20	29.41 ± 5.34 *
Geodia sp.	118.35 ± 1.81	111.57 ± 0.41	91.87 ± 5.12
Thorectandra sp.	89.83 ± 1.65	95.79 ± 9.30	99.62 ± 1.27
Spheciospongia sp.	92.69 ± 1.39	114.92 ± 2.81	92.08 ± 0.71
Esperiopsidae	93.78 ± 5.14	89.50 ± 0.34	76.70 ± 5.41
psammoclema sp.	84.54 ± 2.25	90.77 ± 7.80	80.07 ± 2.20
Sycon sp.	98.47 ± 0.61	104.70 ± 1.66	89.59 ± 1.18
Callyspongia sp.	95.73 ± 1.65	87.13 ± 0.60	12.47 ± 2.15 *
Euryspongia sp.	90.90 ± 4.22	98.38 ± 3.53	89.92 ± 1.26
Strongylacidon sp. 1	93.54 ± 2.31	87.10 ± 3.57	15.36 ± 6.35 *
Clathria sp. 2	96.86 ± 3.48	85.03 ± 3.55	41.94 ± 7.84 *
Chalinula sp.	88.59 ± 6.91	57.36 ± 3.21	10.62 ± 0.36 *
Chondropsis sp. 2	95.49 ± 4.02	56.29 ± 9.25	14.85 ± 1.67 *
Crella sp.	103.06 ± 9.71	92.26 ± 4.92	55.59 ± 2.35
Chondropsis sp. 3	84.54 ± 3.59	62.74 ± 3.27	11.50 ± 1.05 *
Strongylacidon sp. 2	117.64 ± 3.22	117.01 ± 7.18	90.06 ± 5.90
Tethya sp.	103.93 ± 5.77	52.81 ± 3.88	25.80 ± 1.44 *
Chondropsis sp.4	104.24 ± 7.19	75.12 ± 2.37	16.23 ± 4.74 *

Note: In 96-well plates for 48 h exposure to 30 marine sponge crude extracts (at dose of 0.5, 5, 50 μg/ml). Data are shown as relative surviving cell numbers percentage compare to the vehicle control (untreated cells, 0 μg/ml) and are mean of three replicates ± S.E.M. *Significantly different from vehicle control (0 μg/ml) $P < 0.05$.

2.3.3 Secondary screening

2.3.3.1 Anti-proliferation effects of three marine sponge crude extracts on T47D cells

Crude extracts of the selected three sponge extracts (*Tedania cf. anhelans*, *Chondropsis* sp. 1 and *Aplysilla rosea*) were evaluated by the crystal violet assay (relative cell number) on breast cancer cell line T47D to obtain the dose and time-dependent curves. All three sponge extracts inhibited cell proliferation in a dose-dependent manner. Time-dependent effects become more obvious at higher concentrations. Compared to vehicle control, crude extracts from *Tedania cf. anhelans*, and *Chondropsis* sp. 1 showed significant ($P < 0.05$) differences at the doses of 10 and 20 μg/ml after 24 h treatment, and at all the doses after 48 and 72 h treatments, except at 1.25 μg/ml in 48 h treatment. In contrast, extract from *Aplysilla rosea* only showed a significant effect at the dose of 10 and 20 μg/ml in 48 and 72 h treatments (Figure 2.1).

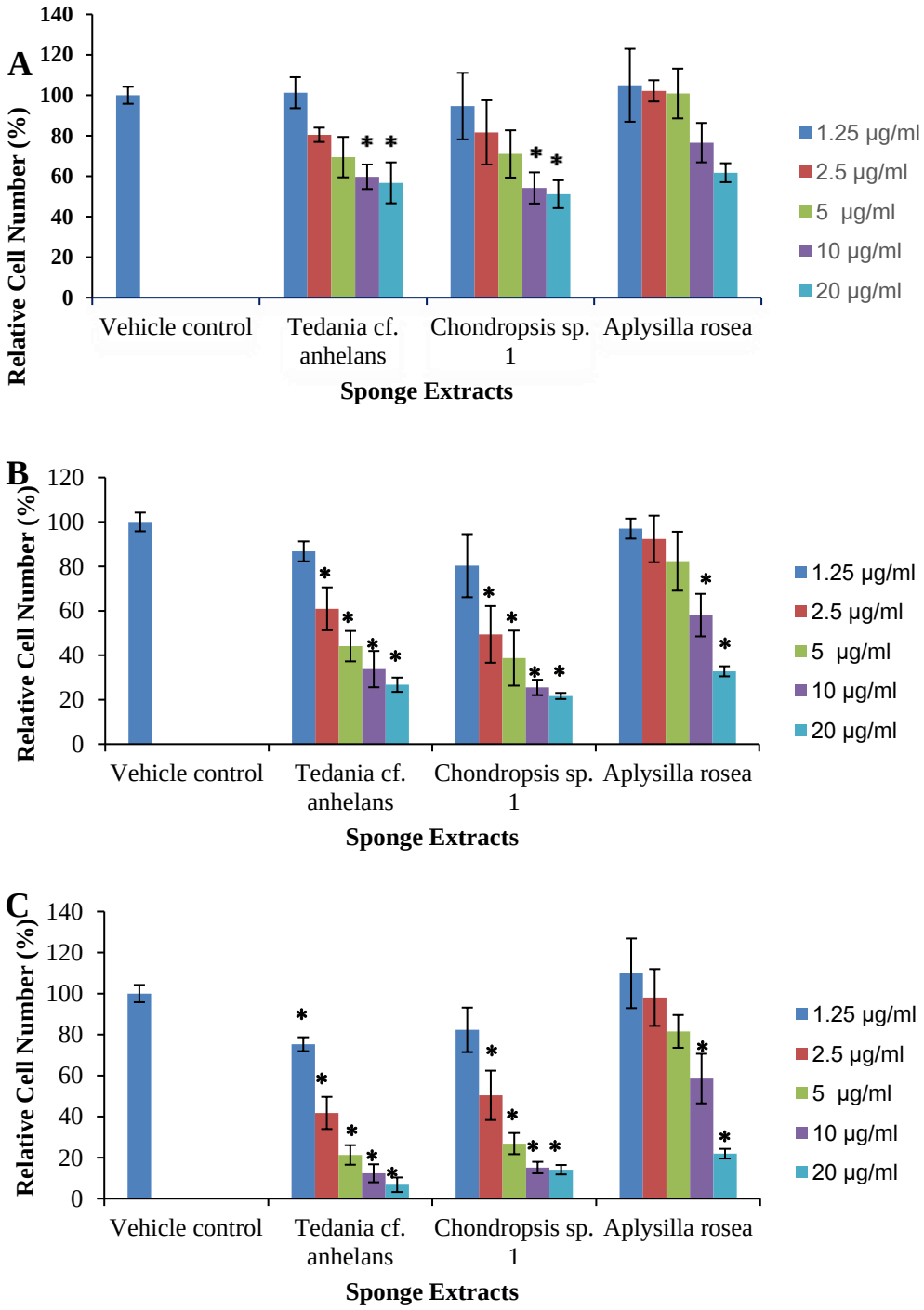


Figure 2.1: Viability of T47D after 24, 48 and 72 h treatment with three sponge extracts.

Time and Dose dependent cytotoxicity was estimated by crystal violet assay in 96-well plates for (A) 24 h, (B) 48 h and (C) 72 h exposure to three marine sponge crude extracts (at dose of 1.25, 2.5, 5, 10 and 20 µg/ml) against human breast cancer cell line T47D. Data are shown as relative surviving cell numbers percentage compared to the medium control (untreated cells, 0 µg/ml) and are mean of three replicates ± S.E.M. * Significantly different from the untreated control at $P < 0.05$.

2.3.3.2 Anti-proliferation effects of three marine sponge crude extracts on MCF7 cells

For MCF7 cells, all three extracts reduced the numbers of viable cells in the dose- and time-dependent manners for crystal violet assay. Crude extracts from *Aplysilla rosea* showed a significant effect at the doses of 10 and 20 μg/ml in 24 h, 48 h and 72 h treatment. Crystal violet assay indicated significantly ($P < 0.05$) reduced survival of MCF7 cells at higher doses (10 and 20 μg/ml) after exposed to extracts of *Tedania cf. anhelans*, and *Chondropsis* sp. 1 at 48 and 72 h compared to vehicle control. There was greater than 90% reduction in cell numbers after 72 h treatment with 10 and 20 μg/ml of extract from *Chondropsis* sp. 1 (80% reduction for *Tedania cf. anhelans*) (Figure 2.2).

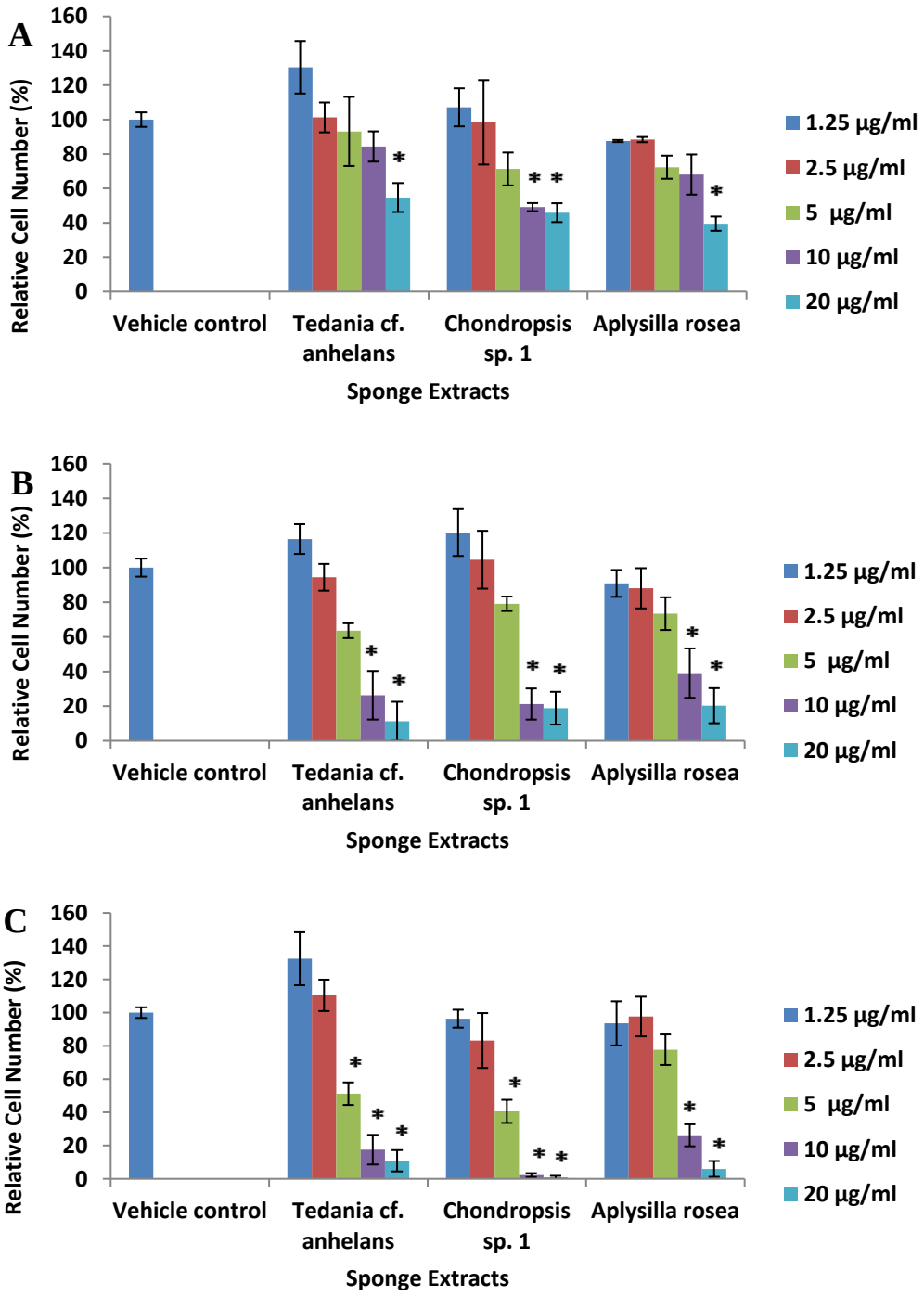


Figure 2.2: Viability of MCF7 after 24, 48 and 72 h treatment with three sponge extracts.

Time and Dose dependent cytotoxicity was estimated by crystal violet assay in 96-well plates for (A) 24 h, (B) 48 h and (C) 72 h exposure to three marine sponge crude extracts (at dose of 1.25, 2.5, 5, 10 and 20 µg/ml) against human breast cancer cell line MCF7. Data are shown as relative surviving cell numbers percentage compared to the medium control (untreated cells, 0 µg/ml) and are mean of three replicates ± S.E.M. * Significantly different from the untreated control at $P < 0.05$.

2.3.3.3 Anti-proliferation effects of three marine sponge crude extracts on MDA-MB-231 cells

Using the crystal violet assay, the evidence of the time- and dose-dependent manners were shown when MDA-MB-231 cells were exposed to three sponge crude extracts (*Tedania cf. anhelans*, *Chondropsis* sp. 1 and *Aplysilla rosea*) at the dose range of 1.25, 2.5, 5, 10 and 20 μg/ml for 24 h, 48 h and 72 h treatments. Compared to vehicle control, crude extracts from *Tedania cf. anhelans*, and *Chondropsis* sp. 1 indicated significant ($P < 0.05$) inhibition at the doses of 5, 10 and 20 μg/ml after 24 and 48 h treatment, and at all the doses after 72 h treatment. In contrast, *Aplysilla rosea* extract only showed a significant effect at the higher dose range of 10 and 20 μg/ml in 48 h against MDA-MB-231 cells, and at the dose range of 5, 10 and 20 μg/ml in 72 h treatment (Figure 2.3).

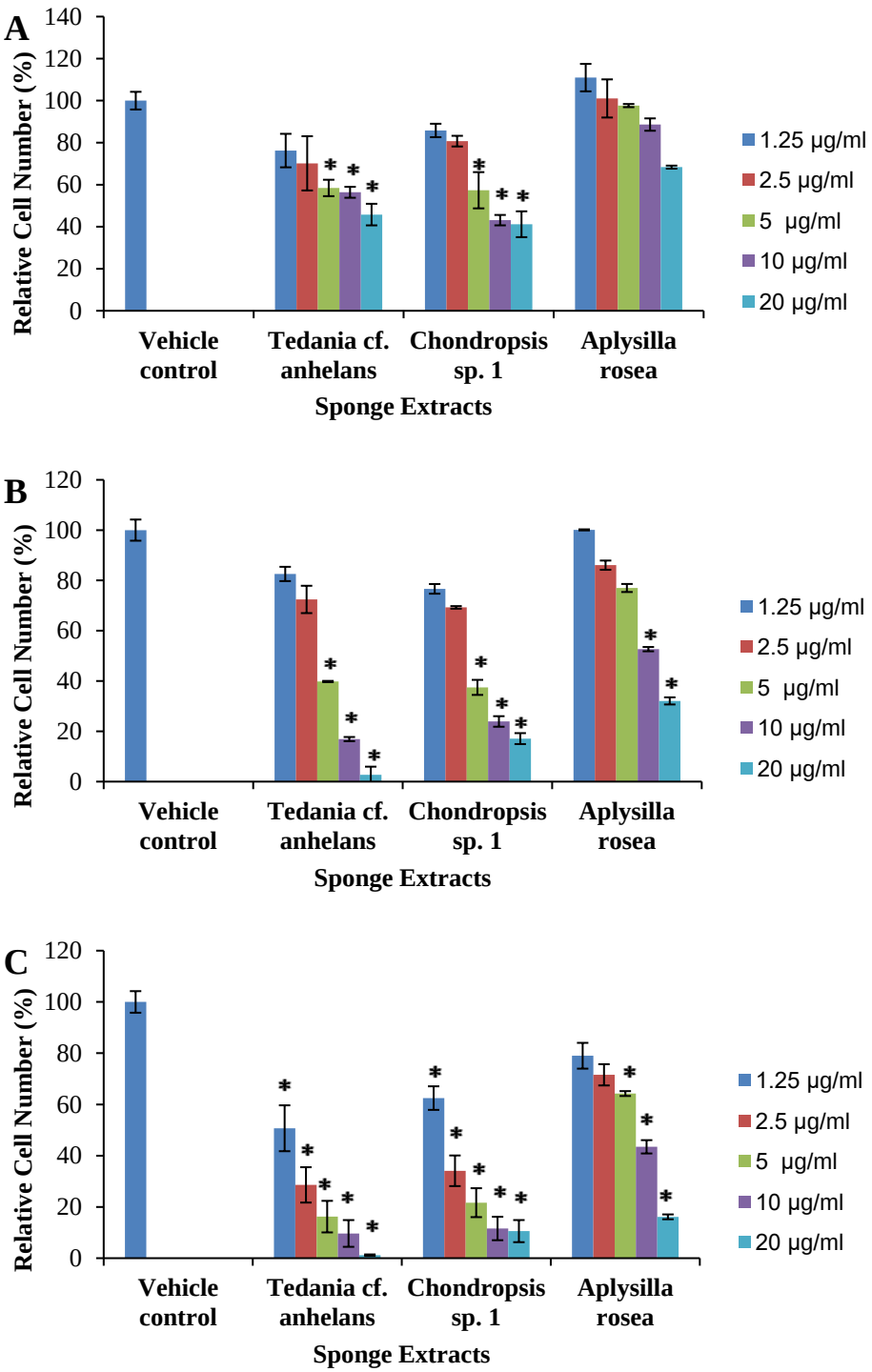


Figure 2.3: Viability of MDA-MB-231 after 24, 48 and 72 h treatment with three sponge extracts.

Time and Dose dependent cytotoxicity was estimated by crystal violet assay in 96-well plates for (A) 24 h, (B) 48 h and (C) 72 h exposure to three marine sponge crude extracts (at dose of 1.25, 2.5, 5, 10 and 20 µg/ml) against human breast cancer cell line T47D. Data are shown as relative surviving cell numbers percentage compared to the medium control (untreated cells, 0 µg/ml) and are mean of three replicates ± S.E.M. * Significantly different from the untreated control at $P < 0.05$.

In contrast, for the crystal violet assay, the three sponge crude extracts caused less cytotoxicity on human non-cancer breast 184B5 cell line, as evidenced by higher IC_{50} value on 184B5 (14.59 μg/ml for *Tedania cf. anhelans*; 11.68 μg/ml for *Chondropsis* sp. 1 and 21.96 μg/ml for *Aplysilla rosea*) compared to the values on T47D, MCF7 and MDA-MB-231. Additionally, for crude extracts from sponge *Tedania cf. anhelans* and *Aplysilla* rosea, there were significant ($P < 0.05$) lower IC_{50} values on all three breast cancer cell lines compared to the values on normal breast cell line. However, the significant differences of IC_{50} values for crude extract (*Chondropsis* sp. 1) were only shown on T47D and MDA-MB-231 cell lines when compared to the value on 184B5 cell line (Table 2.6).

Table 2.6: IC_{50} Value for three human breast cancer cell lines (T47D, MCF7 and MDA-MB-231) and non-cancer breast cell line (184B5) after 48-h treatment with three sponge crude extracts

Sponge	IC_{50} (μg/ml)			
	T47D	**MCF7**	**MDA-MB-231**	**184B5**
Tedania cf. anhelans	5.22 ± 0.89[a]	6.62 ± 1.23[a]	4.05 ± 0.56[a]	14.59 ± 3.23
Chondropsis sp. 1	3.80 ± 0.55[a]	7.28 ± 2.71	4.18 ± 0.21[a]	11.68 ± 2.33
Aplysilla rosea	12.57 ± 0.34[a]	8.31 ± 0.88[a]	11.28 ± 1.14[a]	21.96 ± 2.06

Note: In 96-well plates for 48 h exposure to three marine sponge crude extracts (at dose of 1.25, 2.5, 5, 10 and 20 μg/ml). Data are shown as relative surviving cell numbers percentage compared to the vehicle control (untreated cells, 0 μg/ml) and are mean of three replicates ± S.E.M. The significantly different between cancer cell group and non-cancer cell group showed as [a] $P < 0.05$.

2.3.3.4 Three sponge crude extracts deregulates cell cycle control

The inhibitory effect of three sponge crude extracts on T47D, MCF7 and MDA-MB-231 cells involves the perturbation of cell cycle progression, as detected by flow cytometry analysis. For the crude extract from *Aplysilla rosea*, significant differences ($P < 0.05$) were found in the proportions of cells in the G_0/G_1 phase between treated and untreated cells in all three breast cancer cell lines at the dose of 10 μg/ml (Figure 2.4 – 2.6). In contrast, for the crude extracts from *Tedania cf. anhelans*, the significant differences were only found on T47D cell line in the proportions of cells in the G_0/G_1 (at the doses of 2.5, 5 and 10 μg/ml), S (at the doses of 5 and 10 μg/ml) and G2/M (at dose of 10 μg/ml) phase (Figure 2.4). For the crude extract from *Chondropsis* sp.1, there were significant differences in the T47D cell line with increased proportion of cells in the G_0/G_1 phase and decreased proportion in the S and G_2/M phases at the doses of 5 and 10 μg/ml (Figure 2.4).

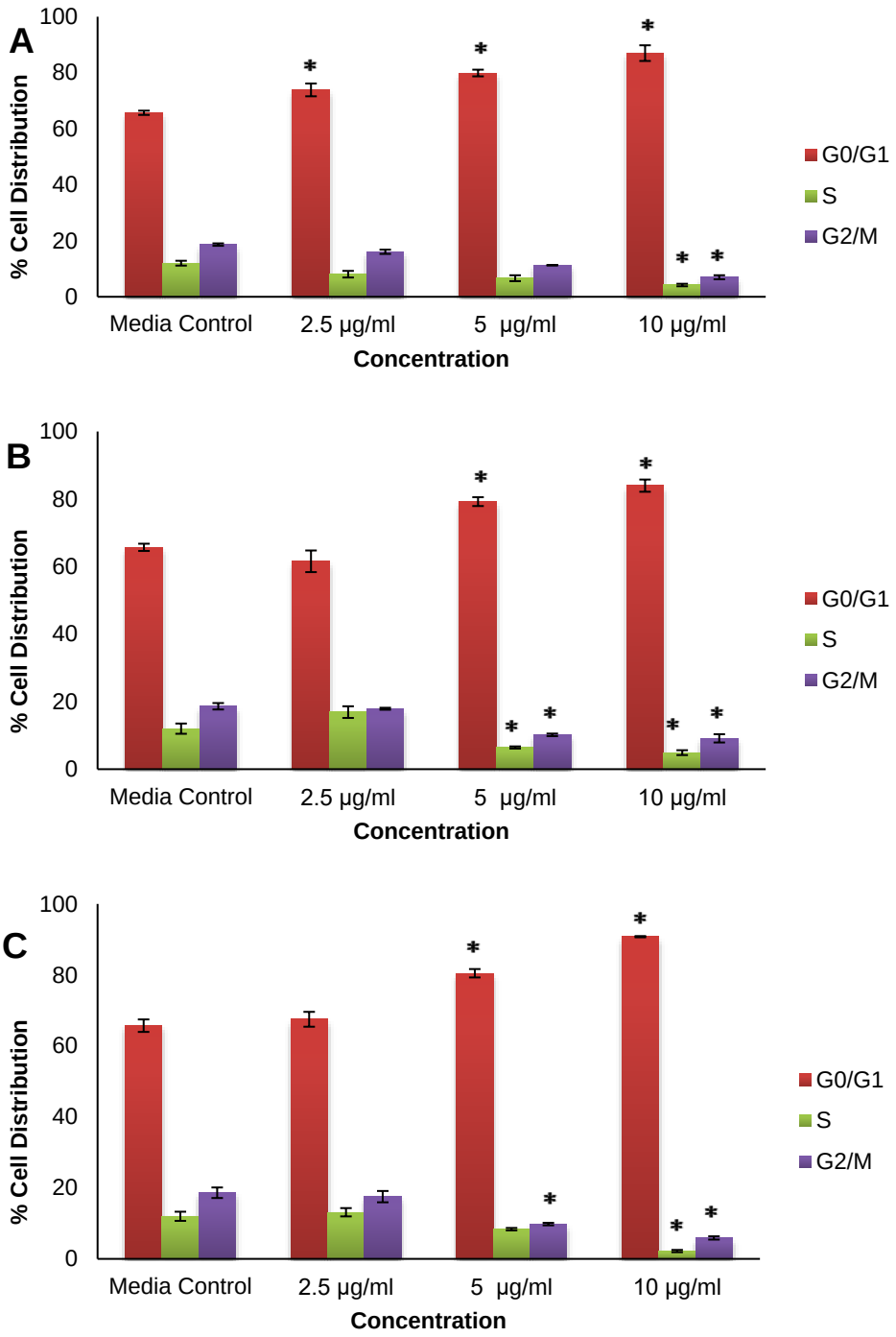


Figure 2.4: Effect of three sponge extracts on T47D cell cycle progression determined by PI staining and analysed for NDA content by flow cytometry.

Note: (A) *Tedania cf. anhelans*, (B) *Chondropsis* sp. 1 and (C) *Aplysilla rosea* Data were obtained from 20,000 events and presented as the percentage of cells in the G_0/G_1, S and G_2/M phases. The data are shown as mean ± S.E.M of three independent experiments. *Significantly different from the untreated control at $P < 0.05$.

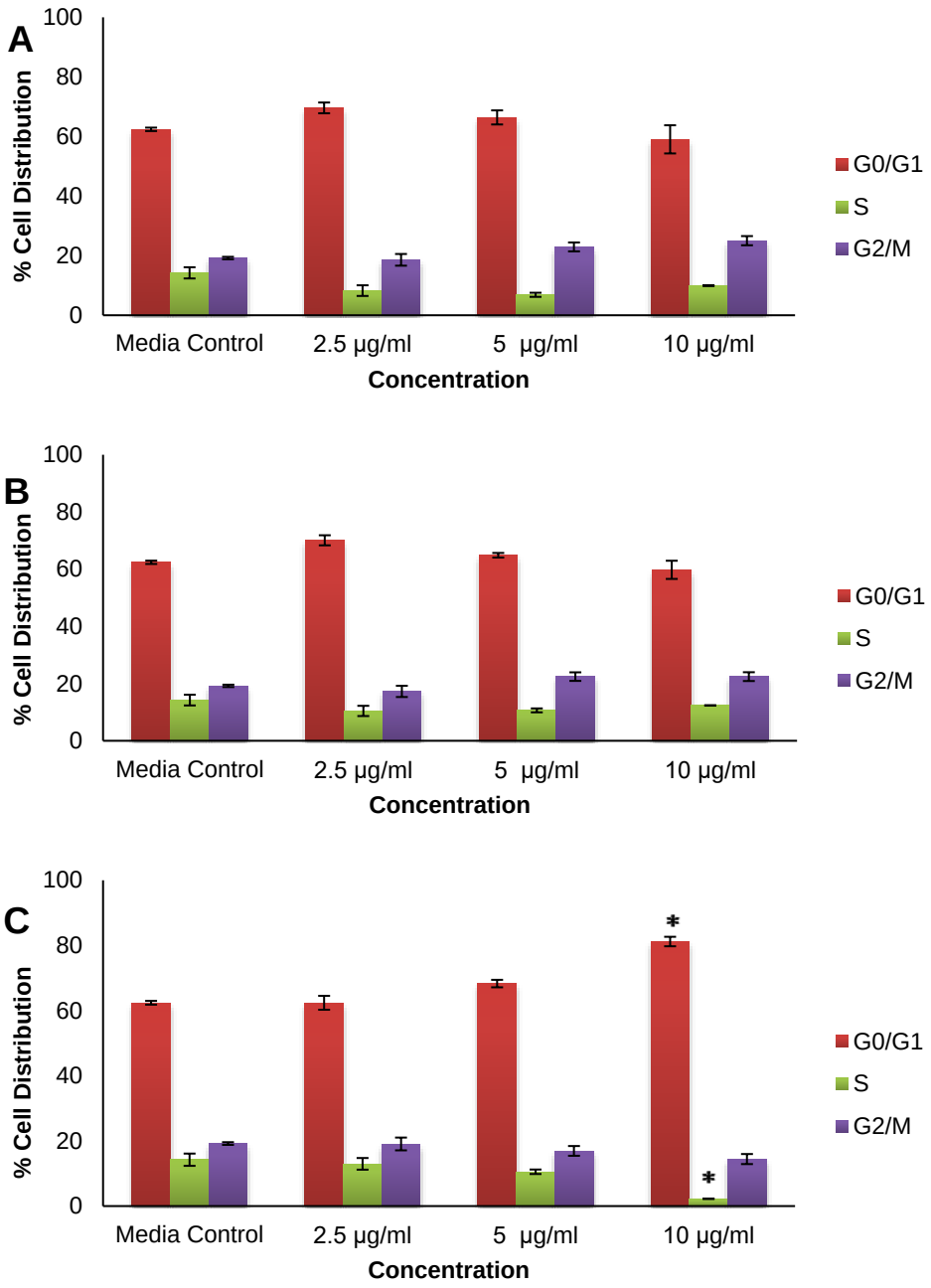


Figure 2.5: Effect of three sponge extracts on MCF7 cell cycle progression determined by PI staining and analysed for NDA content by flow cytometry.

Note: (A) *Tedania cf. anhelans*, (B) *Chondropsis* sp. 1 and (C) *Aplysilla rosea* Data were obtained from 20,000 events and presented as the percentage of cells in the G_0/G_1, S and G_2/M phases. The data are shown as mean ± S.E.M of three independent experiments. *Significantly different from the untreated control at $P < 0.05$.

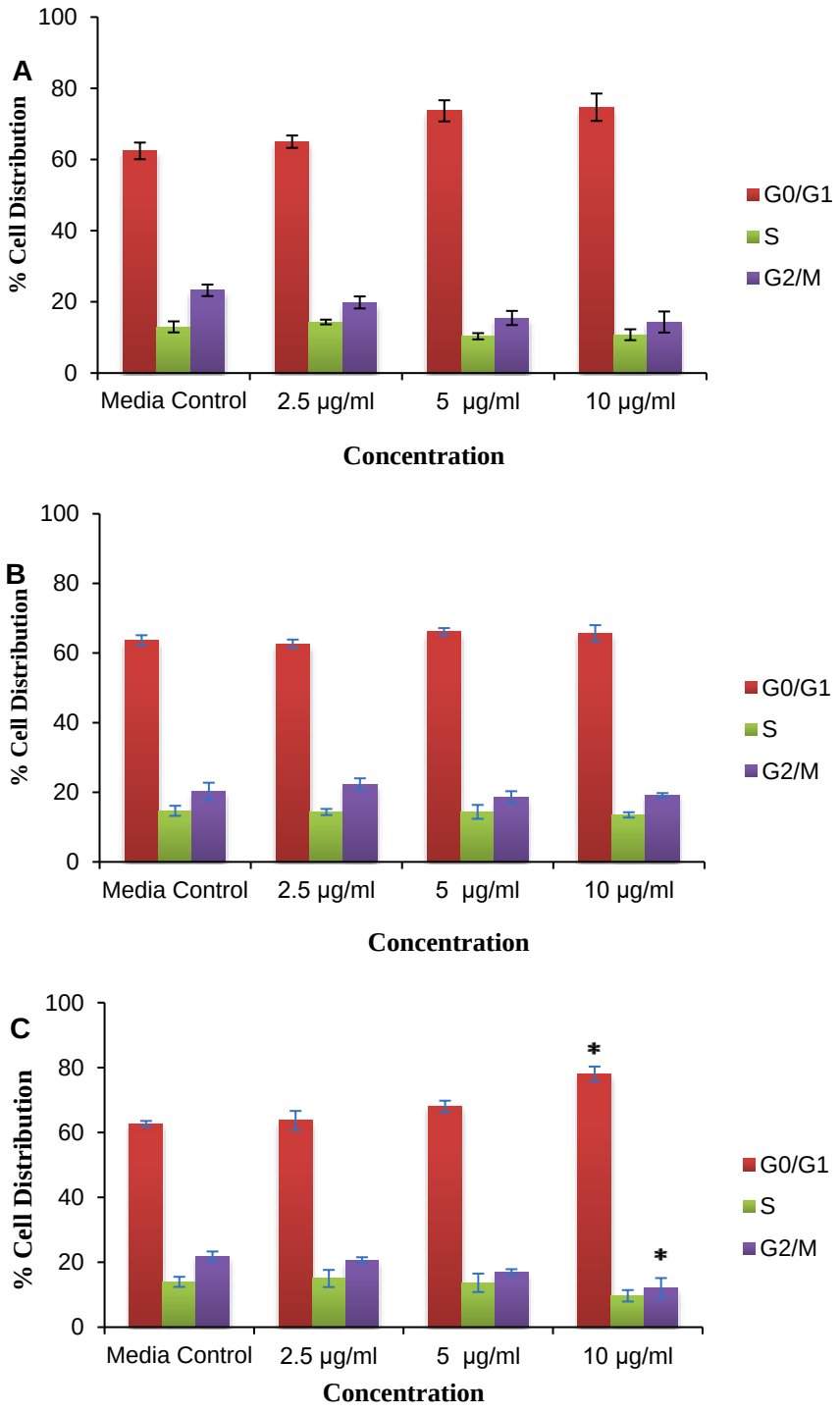


Figure 2.6: Effect of three sponge extracts on MDA-MB-231 cell cycle progression determined by PI staining and analysed for NDA content by flow cytometry.

Note: (A) *Tedania cf. anhelans*, (B) *Chondropsis* sp. 1 and (C) *Aplysilla rosea* Data were obtained from 20,000 events and presented as the percentage of cells in the G_0/G_1, S and G_2/M phases. The data are shown as mean ± S.E.M of three independent experiments. *Significantly different from the untreated control at $P < 0.05$.

2.3.3.5 Three sponge crude extracts induce apoptosis of human breast cancer cell lines

Three breast cancer cell lines after treatments were stained with Annexin V and PI and analysed by flow cytometry to determine whether the decrease in cell viability involved apoptosis. For the crude extracts from *Tedania cf. anhelans* and *Chondropsis* sp.1, the level of induction of apoptosis at the doses of 5 and 10 μg/ml after 48 h treatment were significant ($P < 0.05$) on all three breast cancer cell lines. The highest concentration of crude extracts (10 μg/ml) induced an over 20% increase in early and late apoptosis in all three cancer cell lines, respectively, compared to less than 5% early and late apoptosis in vehicle control cells (Figure 2.7 – 2.9). In contrast, for the crude extract from sponge *Aplysilla rosea*, the significant differences ($P < 0.05$) in the level of induction of early and late apoptosis were only observed at highest dose (10 μg/ml) for all these three cell lines, except for MCF7 cell line, the significant effects ($P < 0.05$) were indicated at a dose of 5 μg/ml for early and late apoptosis (Figure 2.7 – 2.9).

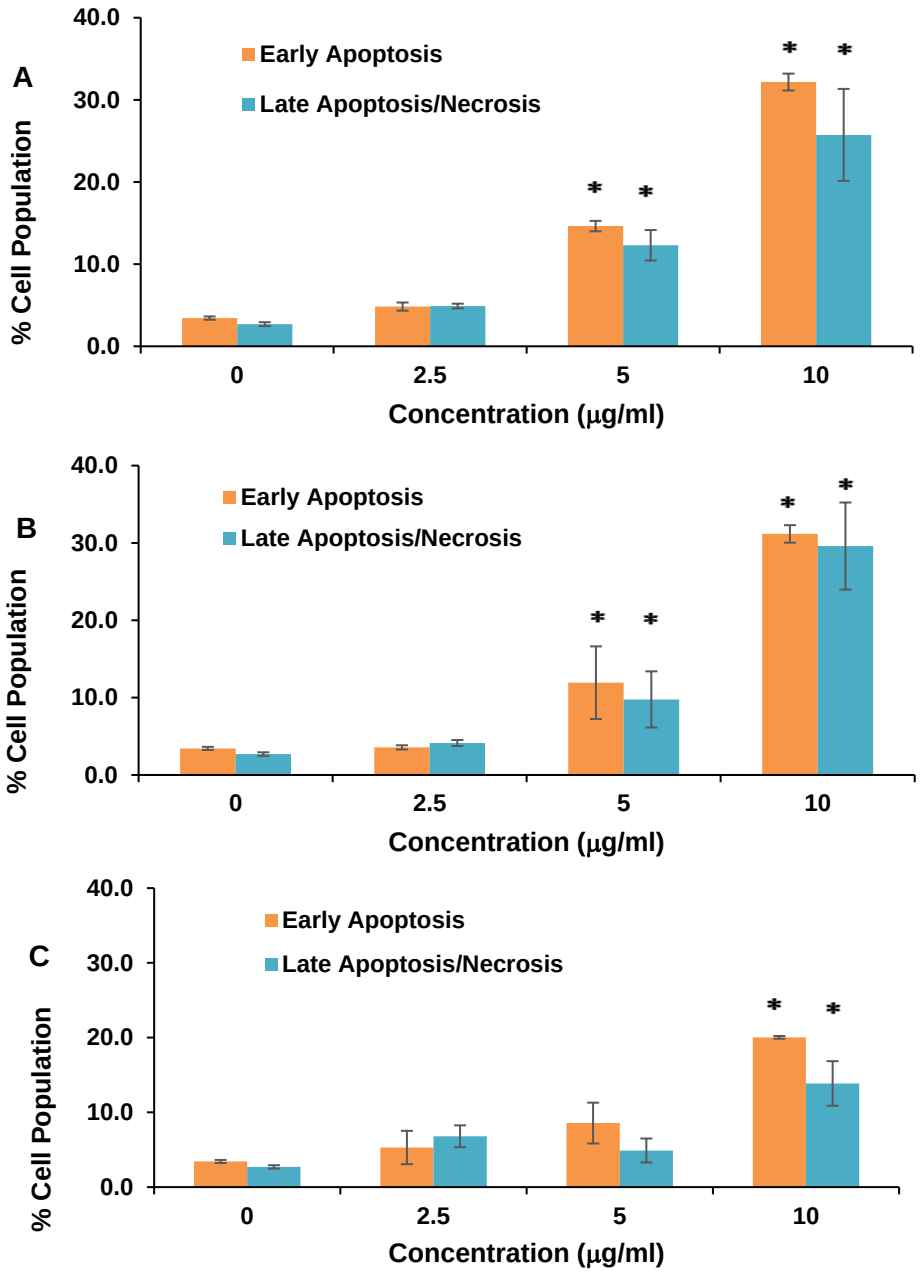


Figure 2.7: Apoptotic effect of three extracts from marine sponges: (A) *Tedania cf. anhelans*, (B) *Chondropsis* sp. 1 and (C) *Aplysilla rosea* on T47D cells determined by flow cytometry

Note: Data were obtained from 20,000 events and early apoptotic cells (Annexin positive) and late apoptotic cells (Annexin positive/PI positive, including necrotic cells) are presented as the percentage of total cells analysed. The data are shown as mean ± S.E.M of three independent experiments. *Significantly different from the untreated control at $P < 0.05$.

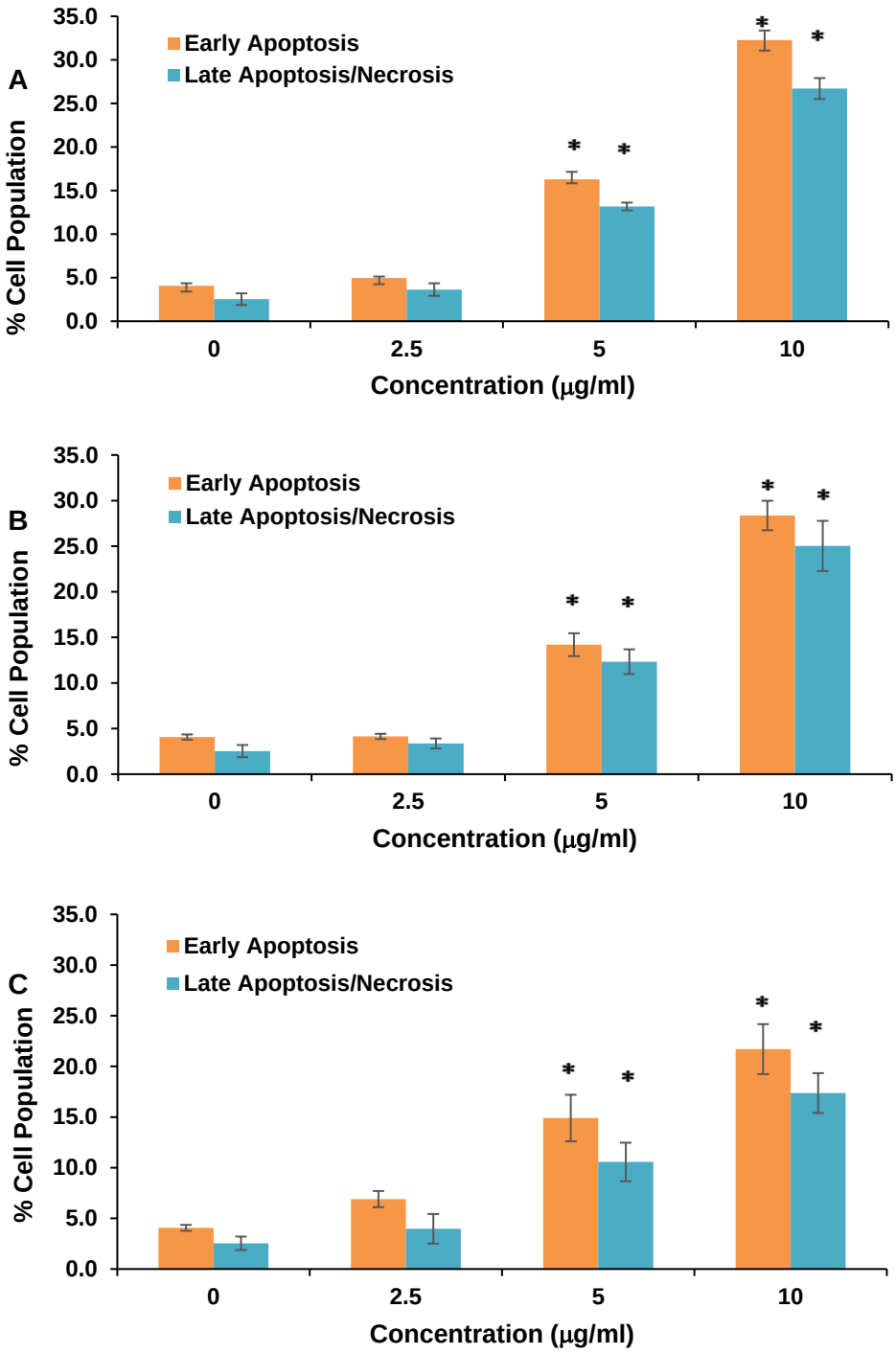


Figure 2.8: Apoptotic effect of three extracts from marine sponges: (A) *Tedania cf. anhelans*, (B) *Chondropsis* sp. 1 and (C) *Aplysilla rosea* on MCF7 cells determined by flow cytometry

Note: Data were obtained from 20,000 events and early apoptotic cells (Annexin positive) and late apoptotic cells (Annexin positive/PI positive, including necrotic cells) are presented as the percentage of total cells analysed. The data are shown as mean ± S.E.M of three independent experiments. *Significantly different from the untreated control at $P < 0.05$.

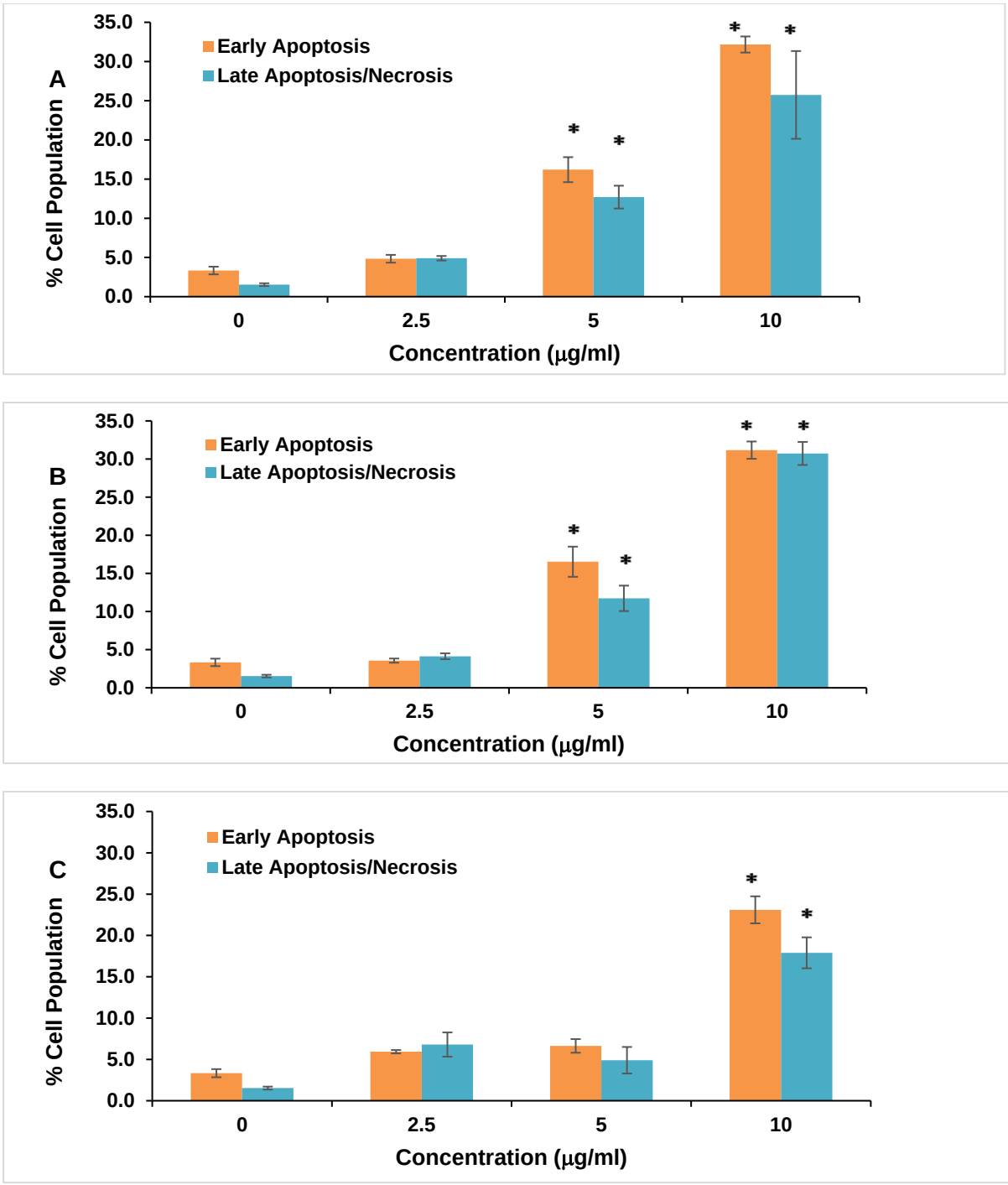


Figure 2.9: Apoptotic effect of three extracts from marine sponges: (A) *Tedania cf. anhelans*, (B) *Chondropsis* sp. 1 and (C) *Aplysilla rosea* on MDA-MB-231 cells determined by flow cytometry

Note: Data were obtained from 20,000 events and early apoptotic cells (Annexin positive) and late apoptotic cells (Annexin positive/PI positive, including necrotic cells) are presented as the percentage of total cells analysed. The data are shown as mean ± S.E.M of three independent experiments. *Significantly different from the untreated control at $P < 0.05$.

2.4 Discussion

In this study, thirty sponge crude extracts were screened for cytotoxicity against three breast cancer cell lines. This study is the first demonstration of the cytotoxicity induced by these 30 South Australian marine sponge extracts on human breast cancer cell lines. Nearly 50% of crude extracts showed the toxic effect at the highest concentration (50 μg/ml) against these three breast cancer cell lines. To date, many cytotoxic, growth inhibitory and other pharmacologically active novel secondary metabolites have been isolated from species/genera of sponges related to these sponge samples tested in this study, as summarised in Table 2.7.

Extracts from *Tedania cf. anhelans*, *Chondropsis* sp.1 and *Aplysilla rosea* showed higher cytotoxic activity against three cancer cell lines in primary screening; therefore, they were chosen for secondary screening. In secondary screening, *Tedania cf. anhelans* crude extract indicated potent inhibition of all three breast cancer cell lines T47D, MCF7 and MDA-MB-231 in the dose-and time-dependent manners.

In the crystal violet assay, these three crude extracts (*Tedania cf. anhelans*, *Chondropsis* sp.1 and *Aplysilla rosea*) reduced 50% of non-breast cancer cell line (184B5) after 48 h treatment with IC_{50} values of 14.6, 11.7 and 21.9 μg/ml, respectively. Interestingly, identical concentrations of these crude extracts exerted higher killing effect on breast cancer cell lines. At the concentration level of 15 μg/ml with 48 h treatment, *Tedania cf. anhelans* extract inhibited breast cancer cells (T47D, MCF7 and MDA-MB-231) growth more than 75, 80 and 90%, respectively. The cell growth inhibitions for extracts from *Chondropsis* sp.1 and *Aplysilla rosea* were 80, 80, and 85%, and 65, 80, 70%, respectively. These findings are encouraging as they support the potential development of therapeutics and natural products from these extracts due to the selectivity against cancer cells.

Table 2.7: literature reports of biological activity related to these tested sponge samples in this study

Species	Compounds	Chemical class	Pharmacological activity	Reference
Suberea sp.	Suberedamines A and B	Alkaloid	Cytotoxicity	(Tsuda et al., 2001)
	Ma'edamines A and B	Alkaloid	Cytotoxicity	(Hirano et al., 2000)
	Lihouidine	Alkaloid	Cytotoxicity	(Bowden et al., 2004)
	19-Hydroxypsammaplysin X	Alkaloid	Cytotoxicity	(Lee et al., 2013)
	Psammaplysin Y			
Psammocinia sp.	Cyclocinamide A	Hexapeptide	Cytotoxicity	(Clark et al., 1997)
	Psymberin	Alkaloid	Cytotoxicity	(Cichewicz et al., 2004)
	Psammocinin A and B	Terpenoid	Cytotoxicity	(Choi et al., 2004)
	Insuetolides A – C	Terpenoid	Cytotoxicity	(Cohen et al., 2011)
	12-deacetoxy-23-hydroxyscalaradial	Terpenoid	Cytotoxicity	(Hahn et al., 2013)
	12-dehydroxy-23-hydroxyhyrtiolide			
	12-*O*-acetyl-16-deacetoxy-23-acetoxyscalarafuran			
	Glyciny-lactams	Sesterterpene	Glycine receptor potentiator	(Balansa et al., 2013)
Ircinia sp.	Ircinals	Alkaloid	Cytotoxicity	(Kondo et al., 1992)
	Haterumalides	Macrolide	Cytotoxicity	(Takada et al., 1999)
	Ircinamine	Alkaloid	Cytotoxicity	(Kuramoto et al., 2002)

Table 2.7: literature reports of biological activity related to these tested sponge samples in this study (continued)

Species	Compounds	Chemical class	Pharmacological activity	Reference
Ircinia sp.	Tedanolides	Macrolide	Cytotoxicity	(Chevallier et al., 2006)
	New metabolites	Sesterterpene	Cytotoxicity	(Issa et al., 2003)
	Kohamaic acids	Sesterterpene	Cytotoxicity	(Kokubo et al., 2001)
	Irciformonins	Terpenoid	Cytotoxicity	(Su et al., 2011)
	Plydiscamides	Alkaloid	Agonists of sensory neuron-specific G protein coupled receptor agonists	(Feng et al., 2007)
	Cheilanthanes	Sesterterpene	Inhibitor of protein kinase	(Buchanan et al., 2001)
Chondropsis sp.	Chondropsins	Macrolide	Cytotoxicity	(Rashid et al., 2001, Cantrell et al., 2000a)
Clathria sp.	Clathrynamides	Amide	Growth inhibitory	(Ohta et al., 1993)
	Mirabilins	Polyketide alkaloid	Cytotoxicity	(El-Naggar et al., 2010)
	Clathsterol	Steroid	Inhibitory of HIV-1	(Rudi et al., 2001)
Aplysilla rosea	Dendrillol-1	Terpenoid	Growth inhibitory	(Betancur-Galvis et al., 2002)
Geodia sp.	Geodiamolides	Peptide	Cytotoxicity	(Tinto et al., 1998, Chan et al., 1987)
Thorectandra sp.	Thorectandramine	Alkaloid	Cytotoxicity	(Charan et al., 2002b)
	Thorectandrols	Sesterterpene	Cytotoxicity	(Charan et al., 2001)
	1-deoxysecofascaplysin A	Alkaloid	Growth inhibitory	(Charan et al., 2004)
Spheciospongia sp.	Spheciosterol sulfates	Steroid	Inhibitor of PKCζ	(Guzii et al., 2008)

Table 2.7: literature reports of biological activity related to these tested sponge samples in this study (continued)

Species	Compounds	Chemical class	Pharmacological activity	Reference
Psammoclema sp.	Trihydroxysterols	Steroid	Growth inhibitory	(Holland et al., 2009)
	Durbinals	Diterpenoid	Cytotoxicity	(Rudi et al., 1995)
Callyspongia sp.	Peroxide-containing acids	Fatty acid	Growth inhibitory	(Toth and Schmitz, 1994)
	Niphatoxin C	Alkaloid	Cytotoxicity	(Buchanan et al., 2007)
	Callyspongiolide	Macrolide	Cytotoxicity	(Pham et al., 2013)
	Hymenialdisine	Alkaloid	Inhibitor of kinase	(Plisson et al., 2014)
Euryspongia sp.	(-)-Frondosins	Sesquiterpene	Inhibitor of HIV	(Hallock et al., 1998)
	Euryspongiols	Steroid	Growth inhibitory	(Dopeso et al., 1994)
	Eurysponginsins	Sesquiterpene	Cytotoxicity	(Yamazaki et al., 2013)
	Eurysterols	Steroid	Cytotoxicity	(Boonlarppradab and Faulkner, 2007)
Crella sp.	Crellastains	Steroid	Cytotoxicity	(D'Auria et al., 1998, Giannini et al., 1999, Zampella et al., 1999)
	Norselic acids	Steroid	Inhibitor of Leishmania parasite	(Ma et al., 2009)

Control of cell cycle progression and apoptosis play crucial role in defence against cancer cells (Senderowicz, 2003, Campbell et al., 2007). The treatment with *Aplysilla rosea* extract on T47D, MCF7 and MDA-MB-231 induced the accumulation of 25%, 19% and 16% of cell population in the G_0/G_1 phase, respectively. Extract from *Aplysilla rosea* showed a less cytotoxic effect and apoptosis induction at the highest dose (10 and 20 μg/ml) compared to the effects of other extracts (*Tenadia cf. anhelans* and *Chondropsis* sp.1). The differences in sensitivity were supported by higher IC_{50} values range of 8.3 to 12.6 μg/ml, compared to 3.80 to 7.28 μg/ml and 4.1 to 6.6 μg/ml for *Tenadia cf. anhelans* and *Chondropsis* sp.1, respectively.

In the literature, four heteroaromatic acids isolated from sponge *Tedania anhelans* (western Gujarat coast of India), and there was no bioactivity study with these compounds (Parameswaran et al., 1997b), and twelve cultivable bacteria isolated from *Tedania anhelans* (Gulang Island, China) showed antimicrobial activities (Zeng et al., 2013). Three new macrolides (Chondropsin A, B and D) were isolated from sponge *Chondropsis* sp (Bass Island, Wollongong, Australia), and Chondropsin A (1587 g/mol) showed potent growth inhibition of tumour cell lines at GI_{50} value of 2.4×10^{-8} M and a range of $> 10^3$ in relative sensitivities of the cell lines (Cantrell et al., 2000b, Rashid et al., 2001). There are twenty-two diterpenoid metabolites isolated from sponge *Aplysilla rosea* (North Bondi, Sydney, Australian), and one compound (dendrillol-1) showed growth inhibition of human cervix cancer cell line (HeLa) and human larynx cancer cell line (Hep-2) at 13 and 13 μg/ml, respectively (Betancur-Galvis et al., 2002, Taylor and Toth, 1997, Karuso and Taylor, 1986).

In conclusion, based on all the evidence showed in primary and secondary screening, extract from sponge *Aplysilla rosea* showed the most potent anticancer properties for new compounds discovery. Therefore, sponge *Aplysilla rosea* was chosen for further isolation and purification study.

Chapter 3: Chemical Constituents of South Australian Marine Sponge *Aplysilla rosea*

Based on the results in chapter two, *Aplysilla rosea* is the top candidate sponge to focus for further study. This chapter presents the isolation, purification and structure elucidation of secondary natural products from marine sponge *Aplysilla rosea* .

3.1Materials

Sponge *Aplysilla rosea* was recollected around Rapid Bay (the same collection site as the primary screening) of South Australian in June of 2013 by Dr Jason Tanner and his team (South Australia Research and Development Institute, Australia), and identified by Ms Shirley Sorokin (Centre for Marine Bioproducts Development, Flinders University, Australia).These sponge materials were transported immediately after collection by keeping them immersed in seawater in the buckets without exposure to air. The fresh sponges were freeze-dried and stored at -80°C until use.

3.2Methods

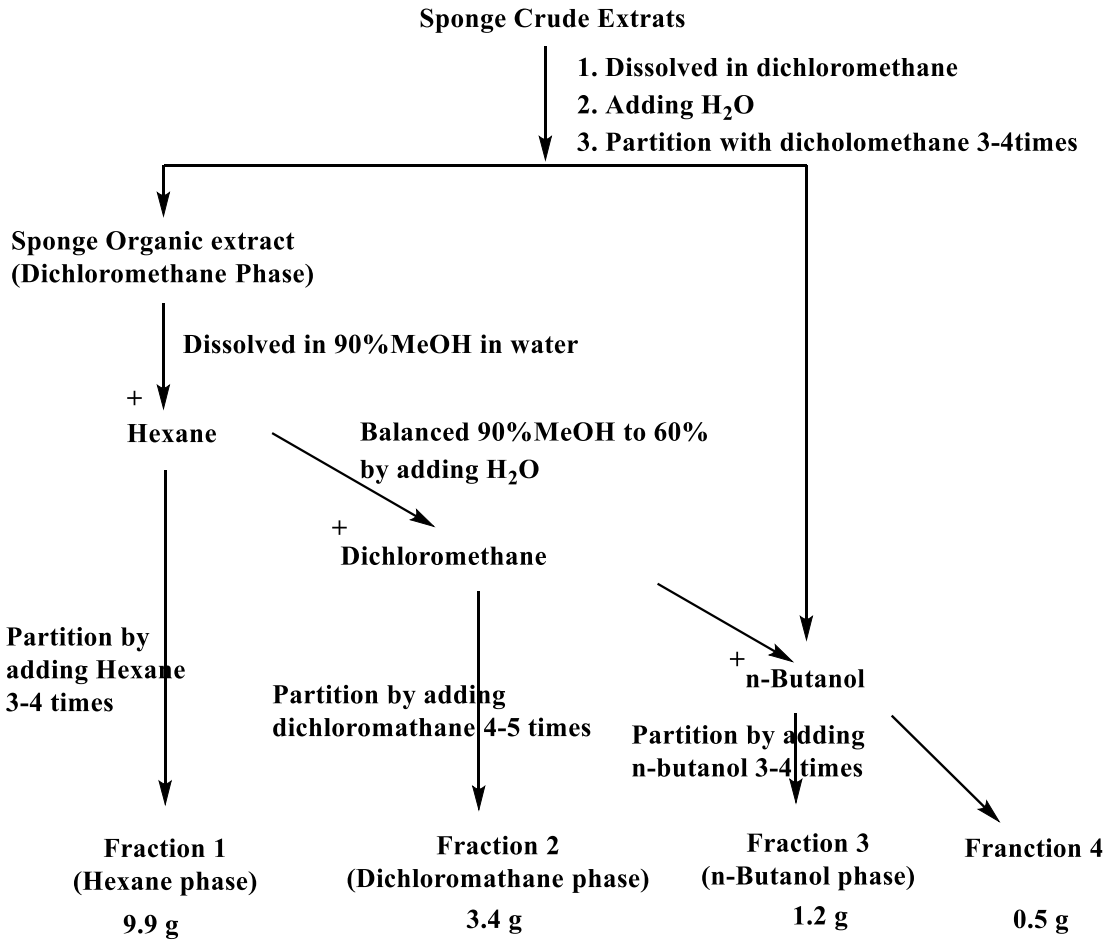
3.2.1 Extraction of marine sponge

Marine sponge *Aplysilla rosea* (200g, dry weight, after freeze-dried) were extracted with methanol and chloroform (1000 ml 1:1, v/v Merck, Germany) at room temperature for four times. The extract was concentrated under reduced pressure at 30°C using a rotary evaporator (EYELA, Japan) to remove organic solvents and give 15g crude extract.

3.2.2 Solvent-solvent partition

Solvent-solvent partition is a broadly employed technique for the separation of organic compounds from a mixture. It involves partition of compounds in two immiscible solvents. Since the technology was based on selective an unequal distribution of solutes between two solutions with a different polarity, the solutes will be more soluble in one solvent compared to the other.

In this study, the solvent-solvent partition was used for the first step in the whole separation process. It was used to separate the extract by polarity into hexane, dichloromethane, *n*-butanol and methanol-water mixture (Ebada et al., 2008b) as follow.



3.2.3 Isolation and purification of secondary metabolites from *Aplysilla rosea*

As bioassay-guided isolation, fraction 1, 2 and 3 were tested for cytotoxicity against two human breast cancer cell lines MCF7 and T47D (Table 3.1). Fractions 1 and 2 showed cytotoxicity against two breast cancer cell lines with IC_{50} value lower than 13 μg/ml, and have similar cytotoxicity against MCF7 cell line. In addition, fractions 1 and 2 have similar characteristic profile in Thin-layer chromatography (TLC) in two different solvent systems (see Appendix A2). Therefore, fractions 1 and 2 were combined together as fraction AR for further isolation and purification of secondary metabolites in this study.

Table 3.1. Cytotoxicity of fraction1, 2 and 3

Extracted phase	cell line (IC50 μg/ml)	
	MCF-7	T47D
Fraction 1	11.93 ± 1.6	12.36 ± 2.4
Fraction 2	12.73 ± 0.4	5.13 ± 2.3
Fraction 3	95.23 ± 3.1	>100

Note: In 96-well plates for 48 h treatment (at dose of 2.5, 5, 10, 20, 50 and 100 μg/ml) against MCF-7 and T47D. Data are the mean of three replicates ± S.E.M.

The fraction AR was then subjected to Sephadex LH-20 column chromatography (CC) with CH_2Cl_2/MeOH (1:1) as eluting solvent to afford three sub-fractions (AR1-AR3), and the TLC profiles of sub-fractions AR 1-3 are shown in Appendix A2.

Sub-fraction AR-3 was separated by column chromatography on (Octadecylsilyl) ODS (50 μm) eluting with MeOH-H_2O (1:5 - 1:0) to give 26 fractions (Fr.AR-3A — AR-3Z), and the TLC profile of fractions AR-3A – 3Z are shown in Appendix A2.

Fractions AR-3F (82.6 mg), AR-3H (170 mg), AR-3I (186 mg), AR-3K (180 mg), AR-3L (230mg), AR-3S (200 mg), AR-3T (214 mg), AR-3U (452 mg) and AR-3Y (373 mg) were separately isolated by CC on Silica Gel with gradient elution (hexanes-acetone, 20:1 to 1:1) to give sub-fractions AR-3F1 – AR-3F4 (4 fractions), AR-3H1 – AR-3H7 (7 fractions), AR-3I1 – AR-3I8 (8 fractions), AR-3K1 – AR-3K9 (9 fractions), AR-3L1 – AR-3L3 (3 fractions), AR-3S1 – AR-3S8 (8 fractions), AR-3T1 – AR-3T9 (9fractions), AR-3U1 – AR-3U12 (12 fractions) and AR-3Y1 – AR-3Y10 (10 fractions).

Fraction AR-3F3 was purified by reversed-phase HPLC with an elution of 60% MeCN detected at the wavelength of 200 nm to give compound **1** (2.0 mL/min, t_R = 18 min, 10.6 mg). Fraction AR-3H5 was isolated by reversed-phase HPLC eluting with 75% MeCN detected at 200 nm to afford compound **2** (2.0 mL/min, t_R = 14 min, 8.7 mg). The purification of AR-3I5 by reversed-phase HPLC eluting with 75% MeCN detected at 200 nm resulted in the isolation of compound **3** (2.0 mL/min, t_R = 14.7 min, 23.4 mg). Fraction AR-3I7 was chromatographed on reversed-phase HPLC eluting with 78% MeCN detected at 200 nm to give compound **4** (2.0 mL/min, t_R = 11 min, 12.8 mg). Fraction AR-3K8 was subjected to reversed-phase HPLC (75% MeCN) to afford compound **5** (2.0 mL/min, t_R = 17 min, 53.0 mg) and **6** (2.0 mL/min, t_R = 28 min, 2.1 mg) at 200 nm. Compound **7** (200 mg) was obtained by CC on silica gel eluting in a stepwise manner with hexane/acetone (10:1 – 1:1) from Fr.AR-L. Fraction AR-3S4 was purified by reversed-phase HPLC with an elution of 80% MeCN detected at the wavelength of 200 nm to give compound **8** (2.0 mL/min, t_R = 45.5 min, 23.0 mg). Fraction AR-3T2 was isolated by reversed-phase HPLC eluting with 85% MeCN detected at 205 nm to afford compound **9** (2.0 mL/min, t_R = 29.5 min, 12.5 mg). Fraction AR-3T8 was subjected to reversed-phase HPLC using 90% MeCN at 240nm

to give compound **10** (2.0 mL/min, t_R = 35.5 min, 5.2 mg). Fraction AR-3T9 was chromatographed on reversed-phase HPLC (90% MeCN) to afford compound **11** (2.0 mL/min, t_R = 30 min, 15.1 mg), **12** (2.0 mL/min, t_R = 31.5 min, 3.0 mg) and **13** (2.0 mL/min, t_R = 34 min, 2.0 mg). Fraction AR-3U4 was subjected to reserved-phase HPLC with 90% MeCN at 205 nm to give compound **14** (2.0 mL/min, t_R = 15.5 min, 11.2 mg) and **15** (2.0 mL/min, t_R = 25.5 min, 6.0 mg). Fraction AR-3U5 was purified by reversed-phase HPLC with 90% MeCN at 205 nm to give compound **16** (2.0 mL/min, t_R = 32.5 min, 22.0 mg). Fraction AR-3U8 was purified by reversed-phase HPLC with an elution of 80% MeCN at 195 nm to give compound **17** (2.0 mL/min, t_R = 36.5 min, 2.7 mg), **18** (2.0 mL/min, t_R = 38.5 min, 2.1 mg) and **19** (2.0 mL/min, t_R = 40 min, 2.3 mg). Fraction AR-3U11 was subjected to reversed-phase HPLC using 80% MeCN at 205 nm to afford compound **20** (2.0 mL/min, t_R = 35 min, 5.1 mg) and **21** (2.0 mL/min, t_R = 40 min, 2.0 mg). Fraction AR-3Y6 was purified by reversed-phase HPLC with 85% MeOH at 205 nm to give compound **22** (2.0 mL/min, t_R = 14.5 min, 1.1 mg). Fraction AR-3Y7 was chromatographed on reversed-phase HPLC (92% MeOH) at 205 nm to afford compound **23** (2.0 mL/min, t_R = 30.5 min, 3.1 mg). Fraction AR-3Y8 was separated by reversed-phase HPLC with 98% MeCN at 205 nm to give compound **24** (2.0 mL/min, t_R = 32.5 min, 8.2 mg).

The details of the schematic isolation and purification of secondary metabolites from fraction AR by using chromatography techniques were shown as follow.

Figure 3.1 Protocols for secondary metaboltes isolated from fraction AR of sponge *Aplysilla rosea*.

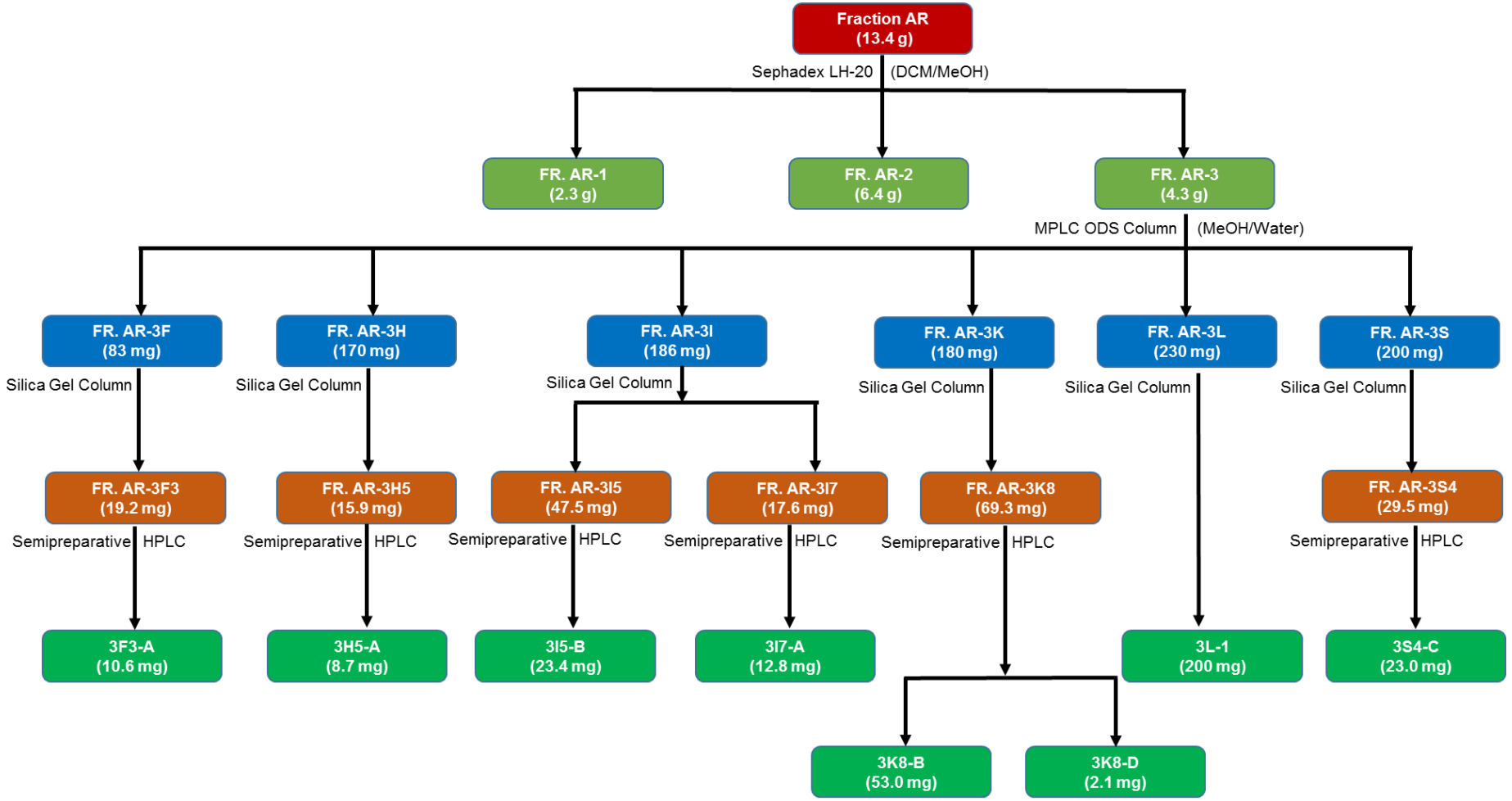


Figure 3.1 Protocols for secondary metaboltes isolated from fraction AR of sponge *Aplysilla rosea* (continued)

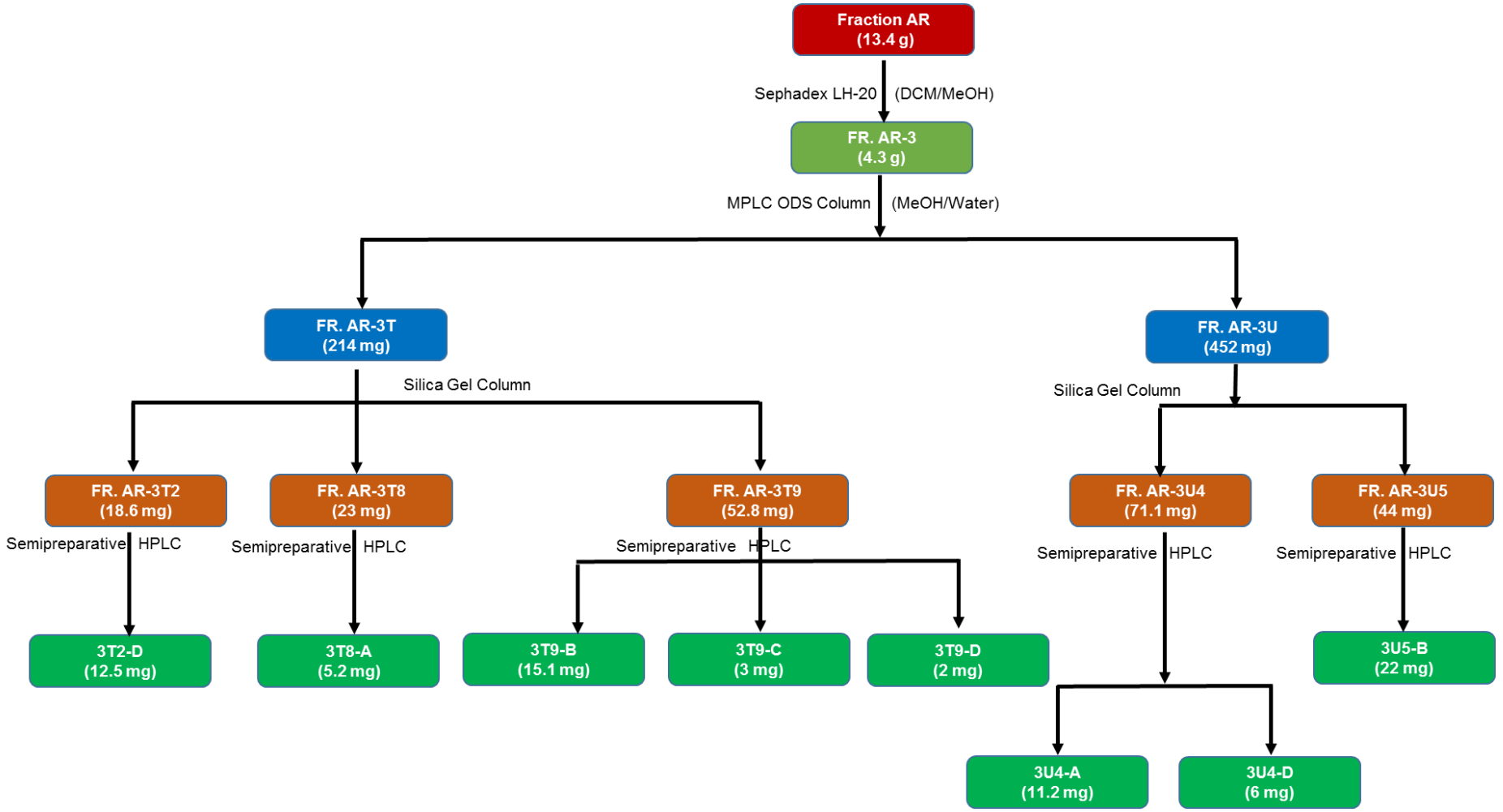
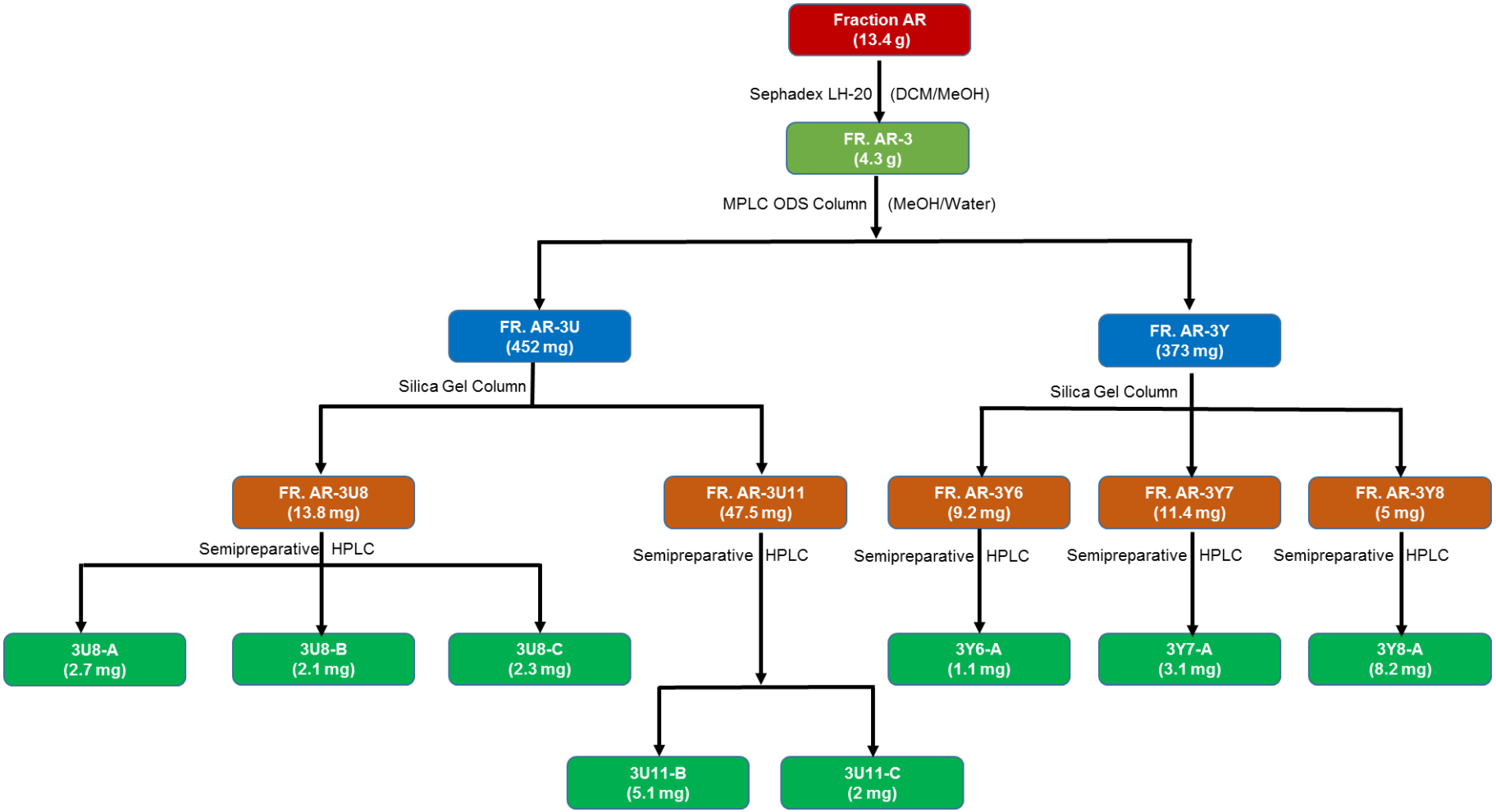


Figure 3.1 Protocols for secondary metaboltes isolated from fraction AR of sponge *Aplysilla rosea* (continued)



3.2.4 Chromatographic methods

3.2.4.1 Thin layer chromatography (TLC)

Thin layer chromatography (TLC) is a straightforward and quick method of chromatography for analysis of the components in the mixtures and the determination of the compound purity. TLC was applied for the detection and monitoring of compounds through the separation processes and was used to optimise solvent systems for column chromatography. It performed on pre-coated TLC plates with silica gel 60 F_{254} (Jiangyou, China). Samples were dissolved in aqueous MeOH of MeOH-DCM (2:1) and loaded drop by drop using glass capillary tube onto TLC plate. The bands were developed with solvent a system (e.g. DCM-MeOH 20:1, 10:1; Hexane-Acetone 10:1, 5:1, 5:2) and separated on the TLC plate. The bands indicated the separation of compounds detected by UV absorbance at 254 nm (fluorescence absorption) and 366 nm (fluorescence), followed by spraying the TLC plates with anisaldehyde-H_2SO_4 reagent and subsequent heating at 180 °C. TLC was conducted pre- and post-experiments to track the identity of each fraction and the qualitative purity of the isolated compounds.

3.2.4.2 Column chromatography (CC)

Flash chromatography is a preparative column chromatography based on an optimised pre-packed column and an eluent at a high flow rate driven by air pressure. It is a straightforward and quick technique widely used for the separation of various organic compounds. In this study, the column, pre-packed by a dried Silica Gel or RP Silica Gel (ODS) (SepaFlash, Santai, China) with different weight (e.g. 12g, 25g, 40g or 80g),

was performed on a medium pressure liquid chromatography (MPLC) machine (PuriFlash 450, up to 50 bar, France). Flash column is filled and saturated with the desired mobile phase just before the sample loading on the top of the column (e.g. Silica Gel column, Hexane-Acetone 20:1; ODS column, 20% MeOH in H_2O). The mobile phase, an isocratic or gradient solvent, is then pumped through the column with the help of air pressure (e.g. Silica Gel column, isocratic solvent Hexane-Acetone 15:1, 12:1, 10:1, 8:1, 6:1, 5:1; ODS column, gradient solvent 20% MeOH to 100% MeOH in H_2O). The flow rate was 10 ml/min for Silica Gel column and 15 ml/min for ODS column.

3.2.4.3 Semi-Preparative high pressure liquid chromatography (HPLC)

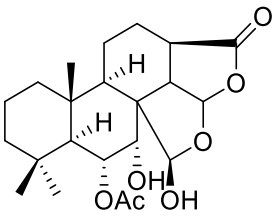
HPLC is a robust, versatile, and usually rapid technique to purify compounds from complex mixtures. The reversed-phase C-18 chromatography was used as the exclusive stationary phase of HPLC in this study. This method was used for the isolation and purification of compounds from fractions previously separated by column chromatography. The most appropriate solvent systems (MeOH-H_2O and MeCN-H_2O) were established before running the HPLC separation. A pre-test must be analysed before all samples for semi-preparative HPLC, and all the conditions must be consistent through the whole preparation time. For example, in the pre-test the best separation option for compound **1** was 60% MeCN (v/v), t_R = 18 min under 200 nm wavelength detected. The whole separation of compound 1 must maintain this condition. Fraction AR-3F3 was dissolved in MeOH (approximately 20 mg/mL), and 50 μL of the sample was injected into HPLC each time, and the flow rate was stabilised at 2 mL/min. The eluted peaks were collected respectively by manual work based on the records of a PDA detector. The collected solution was concentrated and dried by using a rotary evaporator at 30°C.

3.2.5 Nuclear magnetic resonance spectrometry (NMR)

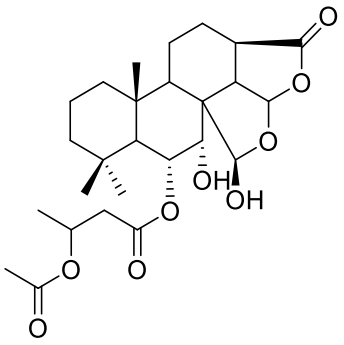
Pure sample (1-5 mg) was dissolved in 0.6 ml of deuterated solvent (Chloroform-D4, DMSO-D6, or Pyrinine-D5, CIL, USA), the choice of which was dependent on the solubility of the samples and the better data collection, and transferred into NMR tube (NORELL, USA) for NMR analysis. NMR spectra were recorded on a Bruker ARX-400/ARX-600 by Dr Ming Zhu, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences. All 1D and 2D spectra were obtained using the standard MestReNava software. Residual solvent signals were used as internal standards (reference signal). The observed chemical shift (δ) values were given in ppm and the coupling constants (J) in Hz.

3.3 Compound list

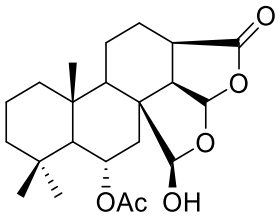
In total, 24-compounds have been structurally identified, with four new compounds labelled with an asterisk.



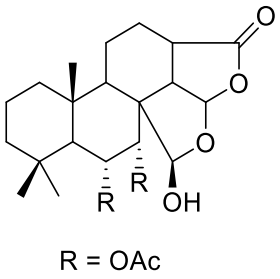
Compound 1 ∗



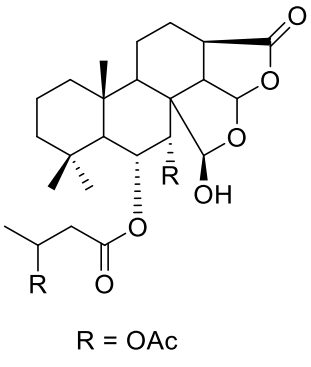
Compound 2 ∗



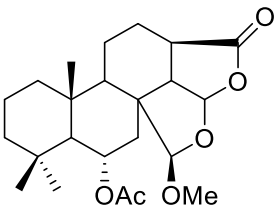
Compound 3



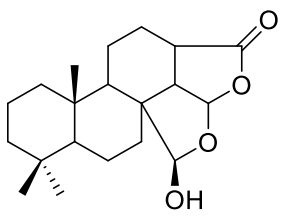
Compound 4



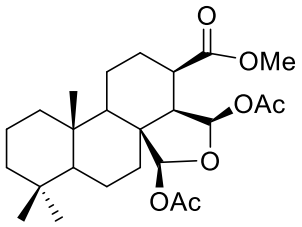
Compound 5 ∗



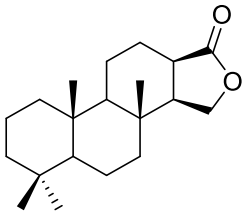
Compound 6 ∗



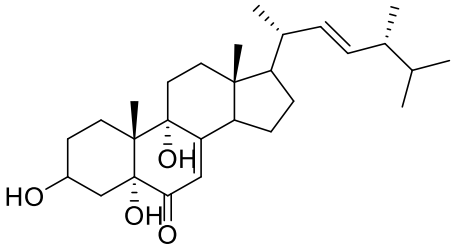
Compound 7



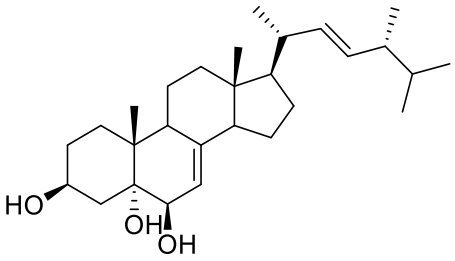
Compound 8



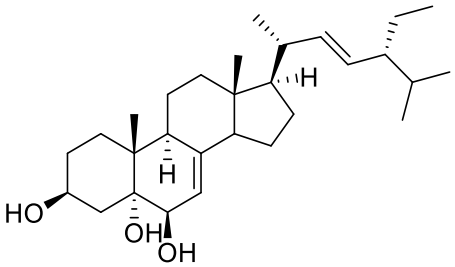
Compound 9



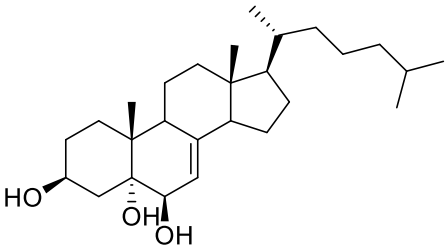
Compound 10



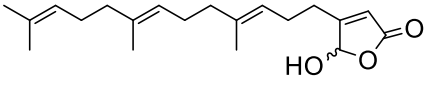
Compound 11



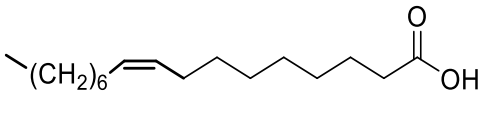
Compound 12



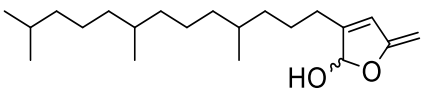
Compound 13



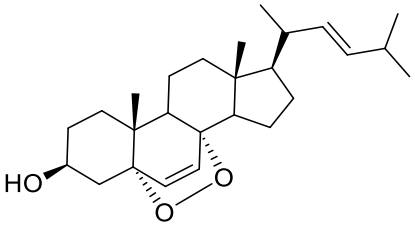
Compound 14



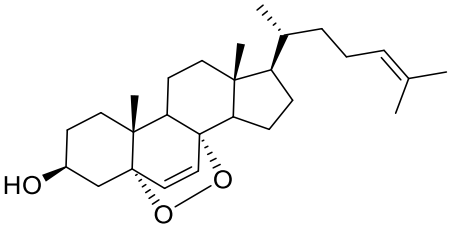
Compound 15



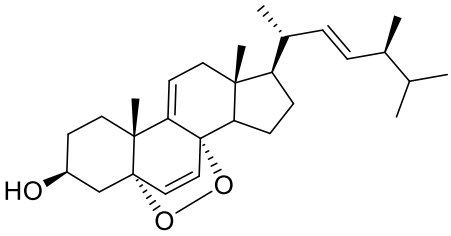
Compound 16



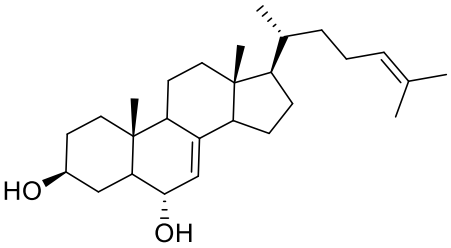
Compound 17



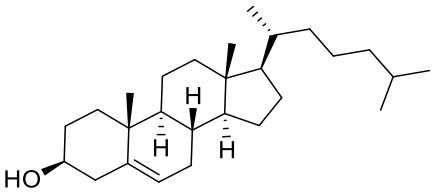
Compound 18



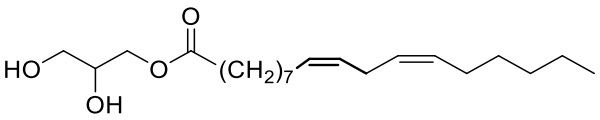
Compound 19



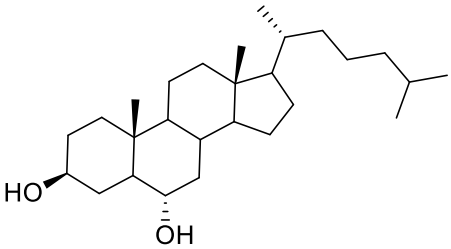
Compound 20



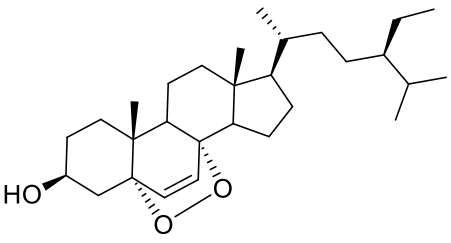
Compound 20



Compound 21



Compound 23



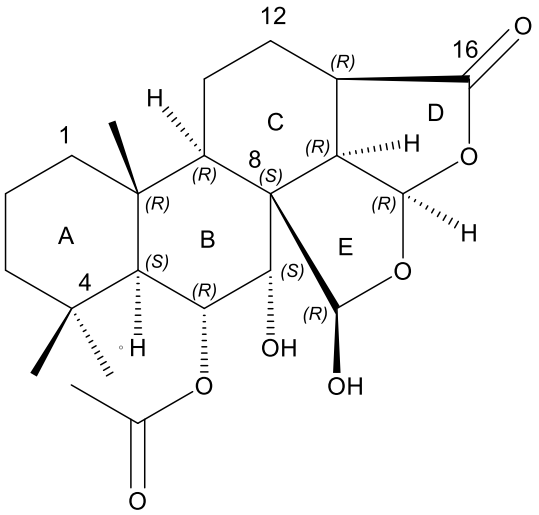
Compound 24

New compound: ∗

3.4 Structural elucidation

3.4.1 Compound 1 (new)

Compound 1: aplyroseol-20	
Biological Source	*Aplysilla rosea*
Sample Code	3F3-A
Sample Amount	10.6 mg
Molecular Formula	$C_{22}H_{32}O_7$
Molecular Weight	408 g/mol
Solubility	MeOH
Physical Description	Colourless crystal



Compound **1**, novel compound, was isolated as a colourless crystal. The molecular formula was determined as $C_{22}H_{32}O_7$ with 7 degrees of unsaturation by HRESIMS ion peak at *m/z* 431.2031 $[M + Na]^+$ ($C_{22}H_{32}O_7$, calcd 431.2043). All 22 carbons were well resolved in the ^{13}C NMR spectrum and were classified by DEPT and HSQC spectra as a lactone (δ_C 178.1), an ester (δ_C 170.5), an acetal carbon (δ_C 104.6), a hemiacetal carbon (δ_C 103.6), three quaternary carbons (δ_C 52.2, 39.6, and 32.9), and four methyl groups (δ_C 36.0, 22.2, 22.0, and 16.9). In the ^1H NMR spectrum, the presence of four sets of coupled protons at H-5 (δ_H 1.84, d, *J* = 12.1 Hz) and H-6 (δ_H 5.35, dd, *J* = 12.1, 1.8 Hz), H-6 (δ_H 5.35, dd, *J* = 12.1, 1.8 Hz) and H-7 (δ_H 3.47, d, *J* = 1.8 Hz), H-13 (δ_H 2.78, dd, *J* = 11.4, 7.8 Hz) and H-14 (δ_H 3.58, dd, *J* = 11.4, 7.8 Hz), and H-14 (δ_H 3.58, dd, *J* = 11.4, 6.0 Hz) and H-15 (δ_H 6.09, d, *J* = 6.0 Hz), one isolated singlet at H-17 (δ_H 5.58, s), two isolated singlets at 7-OH (δ_H 2.60, s) and 17-OH (δ_H 3.71, s) (Table 3.4.1.1). These structural features were similar to those seen in the aplyroseol series of metabolites (Karuso et al., 1984a, Karuso and Taylor, 1986, Taylor and Toth, 1997, Suciati et al., 2011), in this study compound **3**, except there was an additional hydroxyl signal at 7-OH (δ_H 3.71, s).

The HMBC correlations of H_3-20/C-1 and C-10, H-1α/C-2, C-3, and C-10, H_3-19/C-3 and C-4, and H_3-18/C-4 and C-5 established the ring A. Meanwhile, the COSY correlations of H-5/H-6 and H-6/H-7 indicated the connectivity of C_5-C_6-C_7, while the HMBC correlations of H-5/C-6 and C-10, H-6/C-5, H-7/C-6, C-8 and C-9, and H_3-20/C-9 and C-10 suggested the linkage of ring B. The HMBC correlations of H-6/OAc and H-7OH/C-17 revealed the OAc group and 17-OH attached at C-6 and C-7, respectively. The COSY correlations of H_2-11/H_2-12 and H-13/H-14 and HMBC correlations of H-12β/C-9, C-11, and C-14, H-13/C-8, and C-12, and H-11β/C-8, and

C-9 indicated the presence of ring C. The COSY correlations of H-13/H-14 and H-14/H-15 indicated the connectivity of C_{13}-C_{14}-C_{15}, while the HMBC correlations of H-13/C-14 and C-16, H-14/C-16, and H-15/C-16 suggested the linkage of ring D. The presence of ring E was determined by the COSY correlation of H-14/H-15, and HMBC correlations of H-15/C-8, and C-17, and H-17/C-8, and 15. Meanwhile, the HMBC correlation of H-17OH/C-17 indicated the 17-OH attached at C-17.

The NOESY correlations of H-6/H-7, H6-/H_3-19, and H-6/H_3-20 indicated these methyls and protons were positioned on the same face, while the NOESY correlation of H-9/H-13 suggested the two protons were positioned on the other face. Key HMBC, COSY and selected NOESY correlations showed in Figure 3.4.1.1 confirmed this proposal structure.

The X-ray structure of compound 1 (Figure 3.4.1.2) was elucidated using the differences in anomalous dispersion from Cu Kα radiation and allowed unambiguously assignment of the absolute configuration as 5*S*, 6*R*, 7*S*, 8*S*, 9*R*, 10*R*, 13*R*, 14*R*, 15*R*, 17*R* (Crystal data see Table 3.4.1.2 – 5). Following the aplyroseol series, compound **1** is named as aplyroseol-20.

Table 3.4.1.1 NMR data for compound 1

Position	^{13}C (150 MHz) $CDCl_3$	^{1}H (600 MHz) *J* in Hz
1a	39.0 (t)	0.95, m
1b		1.71, dt, (12.7, 3.9)
2a	18.7 (t)	1.46, m
2b		1.60, m
3a	43.3 (t)	1.25, m
3b		1.37, m
4	32.9 (s)	
5	49.7 (d)	1.84, d, (12.1)
6	73.6 (d)	5.35, dd, (12.1, 1.8)
7	72.2 (d)	3.47, d, (1.8)
8	52.2 (s)	
9	47.5 (d)	1.61, m
10	39.6 (s)	
11a	16.5 (t)	1.46, m
11b		1.97, m
12a	23.6 (t)	1.60, m
12b		2.32, m
13	38.0 (d)	2.78, dd, (11.4, 7.8)
14	41.7 (d)	3.58, dd, (11.4, 6.0)
15	104.6 (d)	6.09, d, (6.0)
16	178.1 (s)	
17	103.6 (d)	5.58, s
18	36.0 (q)	0.99, s
19	22.2 (q)	0.87, s
20	16.9 (q)	1.03, s
$OCOCH_3$	170.5 (s)	
$OCOCH_3$	22.0 (q)	2.09, s
7-OH		2.60, s
17-OH		3.71, s

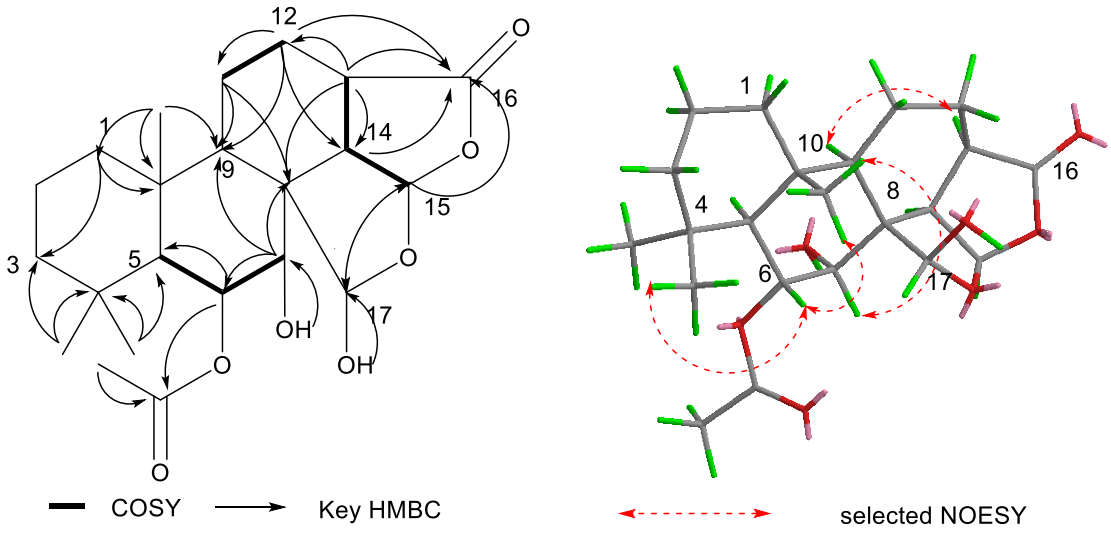
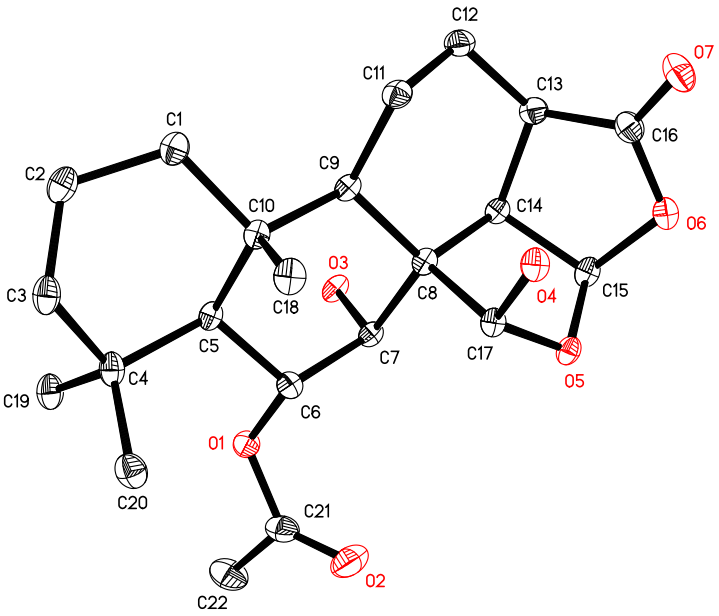
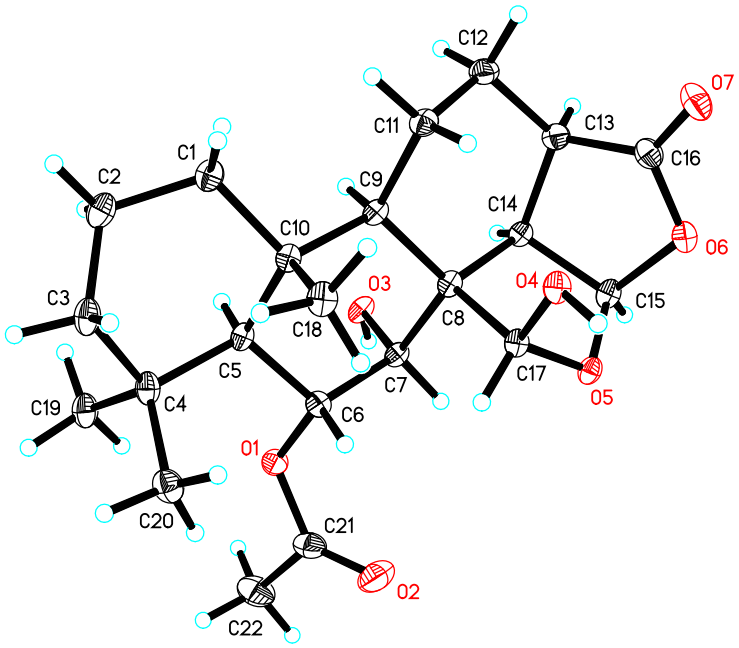


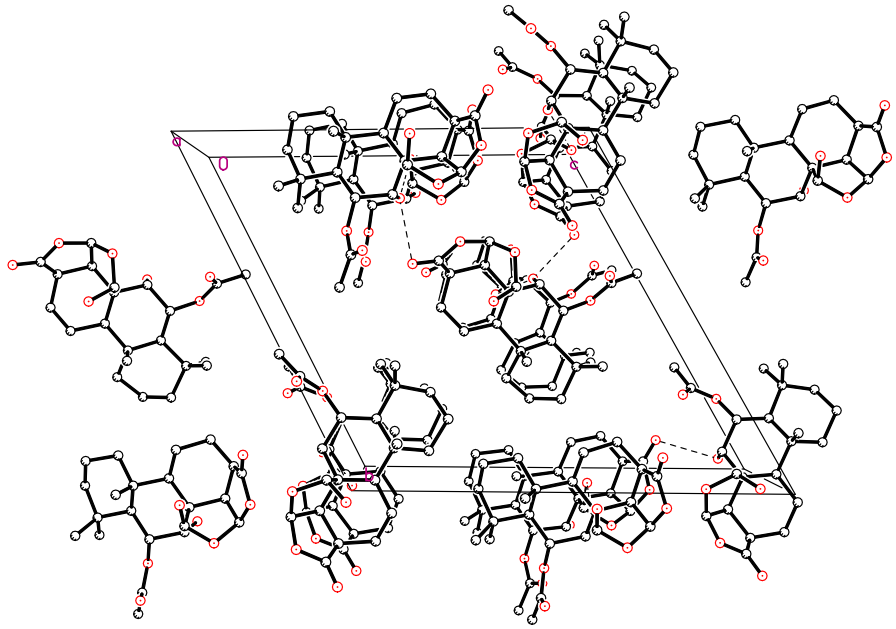
Figure 3.4.1.1. COSY, key HMBC and selected NOE correlations of compound 1.



A



B



C

Fig. 3.4.1.2 ORTEP diagram for compound 1

A: Ortep view of the X-ray structure of compound 1 (hydrogen atoms were omitted for clarity); B: Ortep view of the X-ray structure of compound 1 with hydrogen atoms; C: packing diagram of compound 1.

Table 3.4.1.2 Crystal data of compound 1

Empirical formula	C22 H32 O7	
Formula weight	408.47	
Temperature	130(2) K	
Wavelength	1.54178 Å	
Crystal system	Triclinic	
Space group	P 1	
Unit cell dimensions	a = 13.19810(10) Å	α= 61.7320(10)°
	b = 16.8743(2) Å	β= 84.4090(10)°
	c = 17.2032(2) Å	γ = 76.0090(10)°
Volume	3273.63(7) $Å^3$	
Z	6	
Density (calculated)	1.243 Mg/m^3	
Absorption coefficient	0.756 mm^{-1}	
F(000)	1320	
Crystal size	0.200 x 0.080 x 0.030 mm^3	
R (reflections)	0.0462 (14630)	
wR2 (reflections)	0.1232 (15741)	

Table 3.4.1.3 Positional parameters

Atom	10^4 x	10^4 y	10^4 z
O(1)	-882(2)	-5468(2)	-1968(2)
O(2)	459(3)	-6357(2)	-1009(2)
O(3)	-890(2)	-6010(2)	-3250(2)
O(4)	2548(2)	-5420(2)	-4118(2)
O(5)	2204(2)	-6829(2)	-3070(2)
O(6)	2706(2)	-7104(2)	-4272(2)
O(7)	3051(2)	-6095(2)	-5635(2)
C(1)	-697(3)	-3027(2)	-4985(2)
C(2)	-1550(3)	-2450(3)	-4666(3)
C(3)	-1198(3)	-2496(2)	-3829(3)
C(4)	-1076(3)	-3481(2)	-3045(2)
C(5)	-708(3)	-4217(2)	-3396(2)
C(6)	-171(3)	-5175(2)	-2697(2)
C(7)	57(3)	-5905(2)	-3021(2)
C(8)	779(3)	-5642(2)	-3826(2)
C(9)	250(3)	-4665(2)	-4544(2)
C(10)	-14(3)	-3896(2)	-4243(2)
C(11)	826(3)	-4388(2)	-5427(2)
C(12)	791(3)	-5053(3)	-5785(2)
C(13)	1247(3)	-6061(2)	-5131(2)
C(14)	918(3)	-6370(2)	-4163(2)
C(15)	1889(3)	-7104(2)	-3639(2)
C(16)	2419(3)	-6357(3)	-5082(2)
C(17)	1875(3)	-5838(2)	-3457(2)
C(18)	991(3)	-3661(2)	-4106(2)
C(19)	-2150(3)	-3563(3)	-2630(3)
C(20)	-314(3)	-3591(3)	-2356(3)
C(21)	-463(4)	-6055(3)	-1155(2)
C(22)	-1308(4)	-6250(3)	-481(3)

Table 3.4.1.4 Bond lengths (Å)

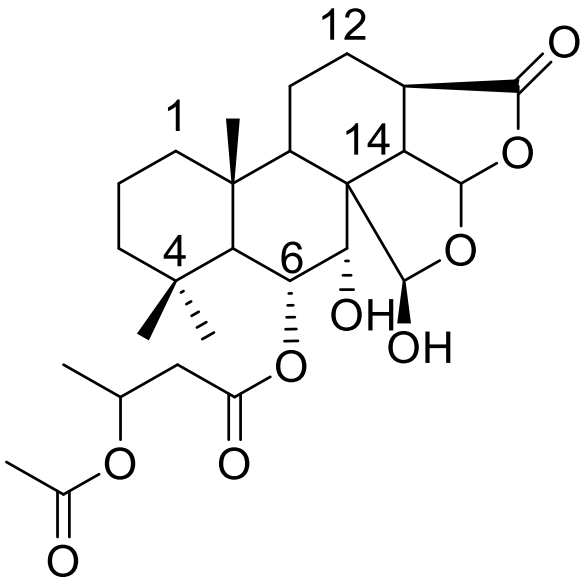
Atoms	Distance	Atoms	Distance
O(1)-C(21)	1.347(5)	O(1)-C(6)	1.451(4)
O(2)-C(21)	1.201(6)	O(3)-C(7)	1.420(4)
O(5)-C(17)	1.442(4)	O(4)-C(17)	1.387(4)
O(6)-C(15)	1.456(4)	O(5)-C(15)	1.396(4)
C(1)-C(2)	1.540(5)	O(6)-C(16)	1.371(4)
C(2)-C(3)	1.518(5)	O(7)-C(16)	1.193(5)
C(4)-C(19)	1.530(5)	C(1)-C(10)	1.551(4)
C(4)-C(5)	1.582(5)	C(3)-C(4)	1.549(5)
C(5)-C(10)	1.577(4)	C(4)-C(20)	1.544(5)
C(6)-C(7)	1.536(5)	C(5)-C(6)	1.526(4)
C(7)-C(8)	1.545(4)	C(8)-C(9)	1.554(4)
C(8)-C(17)	1.535(5)	C(9)-C(11)	1.543(4)
C(8)-C(14)	1.557(4)	C(11)-C(12)	1.525(5)
C(9)-C(10)	1.567(5)	C(13)-C(16)	1.503(6)
C(12)-C(13)	1.532(5)	C(10)-C(18)	1.541(5)
C(13)-C(14)	1.543(4)	C(21)-C(22)	1.504(6)
C(14)-C(15)	1.545(5)		

Table 3.4.1.5 bond angles (degrees)

Atoms	Angle	Atoms	Angle
C(21)-O(1)-C(6)	117.7(3)	C(15)-O(5)-C(17)	108.0(2)
C(16)-O(6)-C(15)	109.9(3)	C(2)-C(1)-C(10)	115.1(3)
C(3)-C(2)-C(1)	110.9(3)	C(2)-C(3)-C(4)	111.3(3)
C(19)-C(4)-C(20)	108.7(3)	C(19)-C(4)-C(3)	107.3(3)
C(20)-C(4)-C(3)	108.4(3)	C(19)-C(4)-C(5)	109.3(3)
C(20)-C(4)-C(5)	113.3(3)	C(3)-C(4)-C(5)	109.6(3)
C(6)-C(5)-C(10)	109.8(3)	C(6)-C(5)-C(4)	114.3(3)
C(10)-C(5)-C(4)	114.1(3)	O(1)-C(6)-C(5)	107.3(3)
O(1)-C(6)-C(7)	106.7(3)	C(5)-C(6)-C(7)	113.3(3)
O(3)-C(7)-C(6)	110.2(3)	O(3)-C(7)-C(8)	109.5(2)
C(6)-C(7)-C(8)	111.2(2)	C(17)-C(8)-C(7)	105.8(2)
C(17)-C(8)-C(9)	121.1(3)	C(7)-C(8)-C(9)	107.5(3)
C(17)-C(8)-C(14)	101.8(3)	C(7)-C(8)-C(14)	108.8(2)
C(9)-C(8)-C(14)	111.2(2)	C(11)-C(9)-C(8)	111.9(3)
C(11)-C(9)-C(10)	113.8(3)	C(8)-C(9)-C(10)	115.3(2)
C(18)-C(10)-C(1)	109.4(3)	C(18)-C(10)-C(9)	110.7(3)
C(1)-C(10)-C(9)	107.3(3)	C(18)-C(10)-C(5)	113.5(3)
C(1)-C(10)-C(5)	106.9(3)	C(9)-C(10)-C(5)	108.8(2)
C(12)-C(11)-C(9)	109.8(3)	C(11)-C(12)-C(13)	112.7(3)
C(16)-C(13)-C(12)	116.0(3)	C(16)-C(13)-C(14)	104.5(3)
C(12)-C(13)-C(14)	115.9(3)	C(13)-C(14)-C(15)	102.8(3)
C(13)-C(14)-C(8)	116.4(3)	C(15)-C(14)-C(8)	103.4(3)
O(5)-C(15)-O(6)	109.5(3)	O(5)-C(15)-C(14)	107.3(3)
O(6)-C(15)-C(14)	107.5(3)	O(7)-C(16)-O(6)	121.0(4)
O(7)-C(16)-C(13)	130.3(4)	O(6)-C(16)-C(13)	108.4(3)
O(4)-C(17)-O(5)	110.6(3)	O(4)-C(17)-C(8)	111.8(3)
O(5)-C(17)-C(8)	102.9(2)	O(2)-C(21)-O(1)	123.5(4)
O(2)-C(21)-C(22)	126.2(4)	O(1)-C(21)-C(22)	110.3(4)

3.4.2 Compound 2 (new)

Compound 2: Aplyroseol-21	
Biological Source	*Aplysilla rosea*
Sample Code	3H5-A
Sample Amount	8.7 mg
Molecular Formula	$C_{26}H_{38}O_9$
Molecular Weight	494 g/mol
Solubility	MeOH
Physical Description	Colourless solid



Compound **2**, novel compound, was obtained as a colourless solid. The molecular formula was determined as $C_{26}H_{38}O_9$ with eight degrees of unsaturation by an HRESIMS ion peak at *m/z* 517.2417 $[M + Na]^+$, and at *m/z* 493.2439 $[M - H]^-$. The NMR data for compound **2** indicated an overall structure similar to compound **1**, with the only difference at the side chain substituted to C-6. These NMR data suggested that the ester group in compound **1** was replaced by a 3-acetoxybutanoate ester group in compound **2**. Briefly, the NMR data of compound **2** indicated three methyl groups (δ_H 1.01, 0.99, and 0.86), a 3-acetoxybutanoate ester (δ_H 5.41, 2.57, 2.03, and 1.30; δ_C 171.4 s, 169.3 s, 67.7 d, 42.9 t, 21.3 q, and 20.6 q), a lactone (δ_C 177.7), and two acetal groups (δ_H 6.04, and 5.58, δ_C 104.5 d, and 103.6 d). In the 1H NMR spectrum, the presence of four sets of coupled protons at H-5 (δ_H 1.89, d, *J* = 12.1 Hz) and H-6 (δ_H 5.30, dd, *J* = 12.1, 2.3 Hz), H-6 (δ_H 5.30, dd, *J* = 12.1, 2.3 Hz) and H-7 (δ_H 3.34, d, *J* = 2.3 Hz), H-13 (δ_H 2.78, dd, *J* = 11.4, 8.0 Hz) and H-14 (δ_H 3.62, dd, *J* = 11.4, 6.2 Hz), and H-14 (δ_H 3.62, dd, *J* = 11.4, 6.2 Hz) and H-15 (δ_H 6.04, d, *J* = 6.2 Hz) (Table 3.4.2).

The COSY correlations of H_2-2'/H-3' and H-3'/H_3-4' indicated the connectivity of C-2'-C-3'-C4', while the HMBC correlations of H_2-2'/C-1', and C-3', H-3'/C-1', C-4', and C-5', H3-4'/C-2', and C-3', and H_3-6'/C-5' suggested the 3-acetoxybuanoate ester. Also, the HMBC correlation of H-6/C-1' indicated that the 3-acetoxybuanoate ester attached at C-6.

The NOESY correlations from H-9 to H-5, H-13, and H_3-18 suggested the three protons and methyl were positioned on the same face, while the NOESY correlations of H-6/H-17OH, H_3-19, and H-20 indicated the two methyls and protons were positioned on the other face.

The key HMBC, COSY and selected NOESY correlations showed in Figure 3.4.2 confirmed this proposal structure. Followed the aplyroseol series, compound **2** was named as aplyroseol-21.

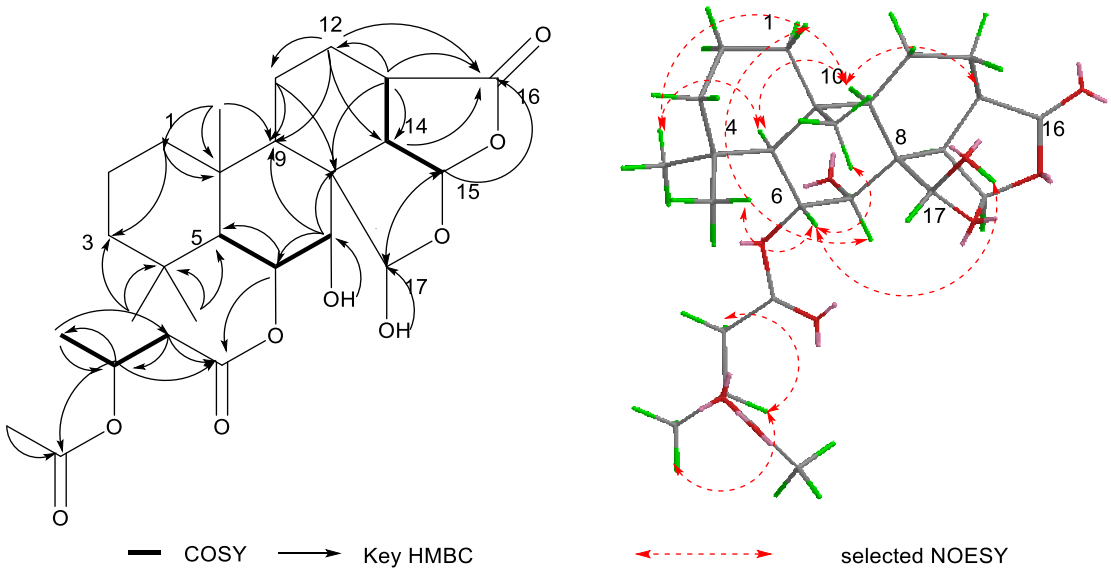


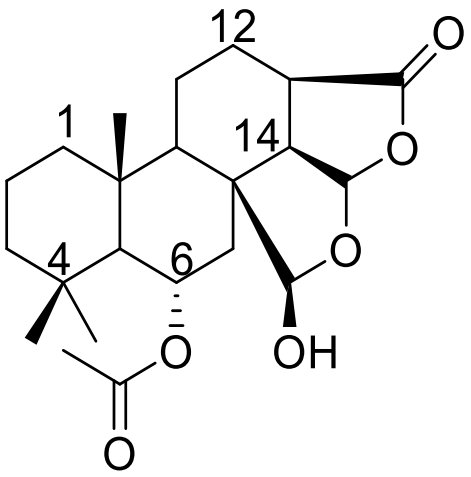
Figure 3.4.2 COSY, key HMBC and selected NOE correlations of compound 2.

Table 3.4.2 NMR data for compound 2

Position	^{13}C (100 MHz) $CDCl_3$	^{1}H (400 MHz) *J* in Hz
1a	39.0 (t)	0.97, m
1b		1.72, m
2a	18.7 (t)	1.47, m
2b		1.58, m
3a	43.3 (t)	1.27, m
3b		1.37, m
4	32.9 (s)	
5	49.6 (d)	1.89, d, (12.1)
6	74.3 (d)	5.30, dd, (12.1, 2.3)
7	71.7 (d)	3.34, d, (2.3)
8	52.3 (s)	
9	47.6 (d)	1.60, m
10	39.6 (s)	
11a	16.6 (t)	1.46, m
11b		1.97, m
12a	23.7 (t)	1.59, m
12b		2.34, m
13	37.9 (d)	2.78, dd, (11.4, 8.0)
14	41.8 (d)	3.62, dd, (11.4, 6.2)
15	104.5 (d)	6.04, d, (6.2)
16	177.7 (s)	
17	103.6 (d)	5.58, d, (1.8)
18	36.1 (q)	0.99, s
19	22.3 (q)	0.86, s
20	16.9 (q)	1.01, s
$OCOCH_2CHCH_3$	169.3 (s)	
$OCOCH_2CHCH_3$	42.9 (t)	2.57, d, (2.0)
$OCOCH_2CHCH_3$	67.7 (d)	5.41, m
$OCOCH_2CHCH_3$	20.6 (q)	1.30, d, (6.4)
$OCOCH_3$	171.4 (s)	
$OCOCH_3$	21.3 (q)	2.03, s
7-OH		3.02, d
17-OH		3.33, d

3.4.3 Compound 3 (known)

Compound 3 : 6α, 17β-dihydroxy-15, 17-oxidospongian-16-one 6, 17-diacetate	
Biological Source	*Aplysilla rosea*
Sample Code	3I5-B
Sample Amount	23.4 mg
Molecular Formula	$C_{22}H_{32}O_6$
Molecular Weight	392 g/mol
Solubility	MeOH, $CDCl_3$
Physical Description	Colourless oil



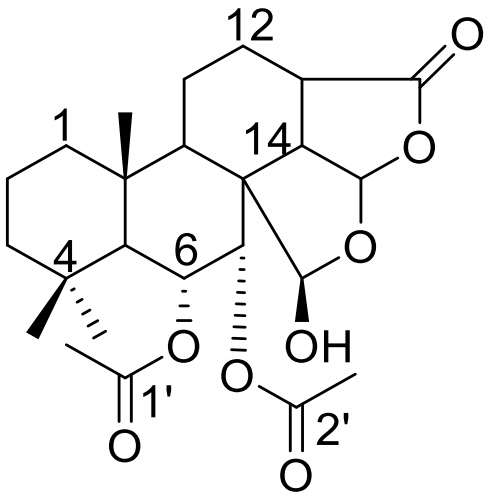
Compound **3** was obtained as a colourless oil by RP-HPLC (Acetonitrile/water, 60/40). Its molecular weight was indicated as 392 g/mol based on the ESI-MS (*m/z* 393.22 [M + H]$^+$). The ^{1}H and ^{13}C NMR spectra showed evidence for four methyl groups (δ_H 0.88, 0.92, 1.00, 2.02), an γ-lactone (δ_C 177.4), an ester (δ_C 170.4), an acetal carbon (δ_C 104.2), a hemiacetal carbon (δ_C 104.0) (Fig. 2.2.3 and Table 2.2.3). The ester identified as an acetate by the presence of a three-proton singlet at 2.02 ppm plus ^{13}C signals at 22.0 (q) and 170.4 (s) ppm (Appendix AR31, AR32 and AR33). Therefore, compound 3I5-B was finally confirmed as 6α, 17β-dihydroxy-15, 17-oxidospongian-16-one 6, 17-diacetate with a molecular formula $C_{22}H_{32}O_6$ by analysis of all data as mentioned above together with comparing the data with reported data (Table 3.4.3) (Ksebati and Schmitz, 1987).

Table 3.4.3 NMR data for compound 3

Position	$CDCl_3$, δ (ppm)	Reference, δ (ppm)
	^{13}C (150 MHz) $CDCl_3$	^{13}C (75 MHz) $CDCl_3$ (Ksebati and Schmitz, 1987)
1	39.2 (t)	39.0 (t)
2	18.7 (t)	18.9 (t)
3	43.3 (t)	43.1 (t)
4	33.5 (s)	33.4 (s)
5	59.1 (d)	58.9 (d)
6	70.5 (d)	70.2 (d)
7	47.4 (t)	47.3 (t)
8	47.5 (s)	47.4 (s)
9	55.0 (d)	54.9 (d)
10	39.9 (s)	39.8 (s)
11	16.9 (t)	16.8 (t)
12	24.0 (t)	23.9 (t)
13	37.9 (d)	37.7 (d)
14	49.7 (d)	49.5 (d)
15	104.2 (d)	104.0 (d)
16	177.4 (s)	177.0 (s)
17	104.0 (d)	103.9 (d)
18	36.2 (q)	36.0 (q)
19	22.0 (q)	21.9 (q)
20	17.4 (q)	17.3 (q)
$OCOCH_3$	170.4 (s)	170.1 (s)
$OCOCH_3$	22.2 (q)	22.0 (q)

3.4.4 Compound 4 (known)

Compound 4: Dendrillol-2	
Biological Source	*Aplysilla rosea*
Sample Code	3I7-A
Sample Amount	12.8 mg
Molecular Formula	$C_{24}H_{34}O_8$
Molecular Weight	450 g/mol
Solubility	MeOH
Physical Description	Colourless needles



Compound **4** was purified as colourless needles that gave a $[M + H]^+$ ion at *m/z* 451 and *m/z* 313 $[M - 2HOAc - H_2O + H]^+$ in the ESIMS, matching the molecular formula $C_{24}H_{34}O_8$ with 8 degrees of unsaturation. The ^1H NMR spectrum of compound **4** was very similar to that of compound **3** except for the presence of an acetate methyl group signal at δ_H 2.19 (3H, q). The ^{13}C spectrum of compound **4** showed the presence of two ester carbonyl signals at δ_C 170.655 (s) and 170.650 (s) ppm. The replacement of two peaks in the methylene region of the ^{13}C NMR spectrum of compound **3** by signals in the ^{13}C NMR spectrum of compound **4** at δ_C 73.5 (d) and 71.2 (d). All other spectral data were consistent with the structural assignment as compound **3**. In additional, the signals for H-6 at δ 5.35 (H, $J_{5\alpha, 6\beta}$ = 12.2 Hz, $J_{6\beta, 7\beta}$ = 2.5 Hz) and H-7 at 4.97 (H, $J_{6\beta, 7\beta}$ = 2.5 Hz) defined the axial α-disposition of the C-7 acetate group and thus a *cis*- and *β*-relationship of C-6 and C-7 protons (Appendix AR41 - AR47). The HMBC correlations of H-6/C-1' and H-7/C-2' indicated the two acetate methyl groups attached C-6 and C-7, respectively. The COSY and key HMBC correlations were showed in Figure 3.4.4. Based on these data and a comparison with data available in the literature (Table 3.4.4), compound **4** was established to be Dendrillol-2 (Karuso et al., 1986, Taylor and Toth, 1997).

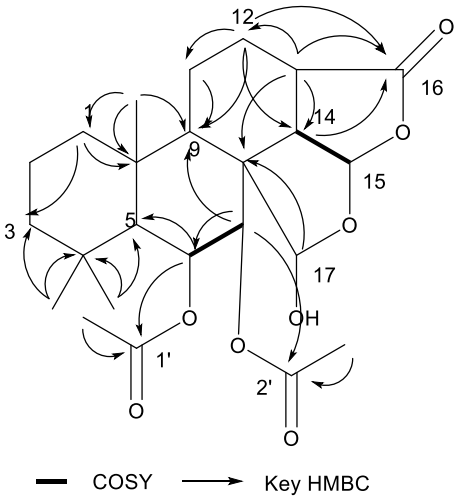


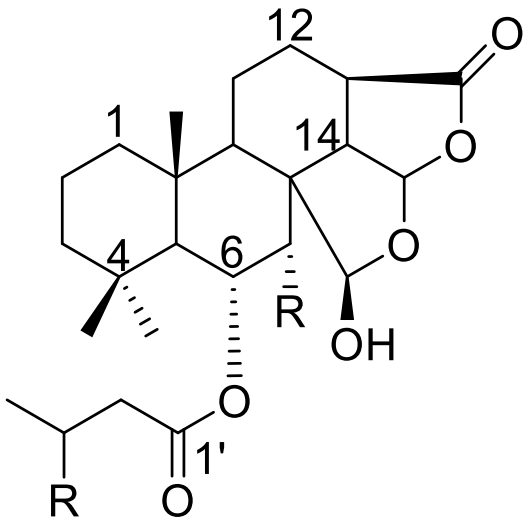
Figure 3.4.4 COSY and key HMBC correlations of compound 4

Table 3.4.4 NMR data for compound 4

Position	$CDCl_3$, δ (ppm), J in Hz		Reference, δ (ppm), J in Hz (Karuso et al., 1986)	
	^{13}C (150 MHz) $CDCl_3$	^{1}H (600 MHz)	^{13}C (75 MHz) $CDCl_3$	^{1}H (300 MHz)
1a	38.8 (t)	0.96 m	38.9 (t)	
1b		1.75 m		
2a	18.5 (t)	1.44 m	18.5 (t)	
2b		1.64 m		
3a	43.1 (t)	1.23 m	43.1 (t)	
3b		1.39 m		
4	32.8 (s)		32.8 (s)	
5	48.9 (d)	1.51 d (12.2)	49.0 (d)	
6	71.2 (d)	5.35 dd (12.2, 2.5)	71.2 (d)	5.35 dd (12.1, 2.5)
7	73.5 (d)	4.97 d (2.5)	73.5 (d)	4.97 d (2.5)
8	51.3 (s)		51.5 (s)	
9	50.8 (d)	1.75 dd (12.4, 2.5)	50.8 (d)	
10	39.4 (s)		39.5 (s)	
11a	16.3 (t)	1.48 m	16.4 (t)	
11b		2.02 m		
12a	23.3 (t)	1.51 m	23.3 (t)	
12b		2.38 m		
13	37.6 (d)	2.74 m	36.7 (d)	2.74 m
14	42.5 (d)	2.74 m	42.6 (d)	2.74 m
15	104.0 (d)	6.02 m	104.0 (d)	6.03 m
16	177.0 (s)		176.8 (s)	
17	103.9 (d)	5.64 br d	103.1 (d)	5.64 br d
18	35.9 (q)	0.97 s	16.7 (q)	0.98 s
19	21.8 (q)	0.86 s	21.8 (q)	0.86 s
20	16.8 (q)	1.04 s	35.8 (q)	1.05 s
$OCOCH_3$	170.655 (s)		170.4 (s)	
$OCOCH_3$	170.650 (s)		170.5 (s)	
$OCOCH_3$	21.1 (q)	2.19 s	21.1 (q)	2.20 s
$OCOCH_3$	21.5 (q)	1.98 s	36.0 (q)	1.99 s

3.4.5 Compound 5 (new)

Compound 5: Aplyroseol-22	
Biological Source	*Aplysilla rosea*
Sample Code	3K8-B
Sample Amount	53.0 mg
Molecular Formula	$C_{28}H_{40}O_{10}$
Molecular Weight	536 g/mol
Solubility	MeOH
Physical Description	Colourless solid



R = OAc

Compound **5,** a new compound, was obtained as colourless solid. The molecular formula was determined as $C_{28}H_{40}O_{10}$ with 9 degrees of unsaturation by an HRESIMS ion peak at m/z 559.2523 $[M + Na]^+$. The ^1H and ^{13}C NMR spectra showed evidence for three methyl groups (δ_H 1.03, s, 0.97, s, 0.86, s; δ_C 16.6, 35.8, 22.3), a 3-acetoxybutanoate ester (δ_H 5.23, 2.49, 1.96, and 1.27; δ_C 170.3 s, 170.2 s, 67.5 d, 41.1 t, 21.1 q, and 19.6 q), an ester carbon (δ_C 170.7 s), an acetate methyl (δ_H 2.17; δ_C 21.0 q), a lactone carbon (δ_C 177.1), and two acetal groups (δ_H 5.99, and 5.58; δ_C 104.5 d, and 103.4 d). These structural features were similar to compound **2**, except the 7-OH in compound **2** was replaced by an ester group in compound **5**, which was supported by the HMBC correlations of H-7 and H-17 to ester carbon (δ_C 170.7 s). Briefly, the NMR data of compound **2** indicated six sets of coupled protons at H-5 (δ_H 1.73, d, *J* = 12.2 Hz) and H-6 (δ_H 5.39, dd, *J* = 12.2, 2.5 Hz), H-6 (δ_H 5.39, dd, *J* = 12.2, 2.5 Hz) and H-7 (δ_H 4.95, d, *J* = 2.5 Hz), H-13 (δ_H 2.72, dd, *J* = 11.4, 6.6 Hz) and H-14 (δ_H 2.68, dd, *J* = 11.4, 5.3 Hz), H-14 (δ_H 2.68, dd, *J* = 11.4, 5.3 Hz) and H-15 (δ_H 5.33, d, *J* = 5.3 Hz), and two sets in the butanoate ester group (δ_H 5.23, m, *J* = 6.3 Hz) with (δ_H 2.49, d, *J* = 6.3 Hz) and (δ_H 1.27, d, *J* = 6.3 Hz) (Table 3.4.5).

The HMBC correlations of H-6/C-1' and H-7/C-2' suggested that the 3-acetoxybutanoate ester and acetal ester attached C-6 and C-7, respectively. The NOESY correlations of H-10a/H-13 and H-15 indicated the three protons were positioned on the same face, while NOESY correlations of H-6/H-17OH, H_3-19, and H_3-20, and H-7/H_3-20 suggested the five protons were positioned on the other face. The key HMBS, COSY and selected NOESY correlations showed in Figure 3.4.5 confirmed this proposal structure. Followed the aplyroseol series, compound **5** was named as aplyroseol-22.

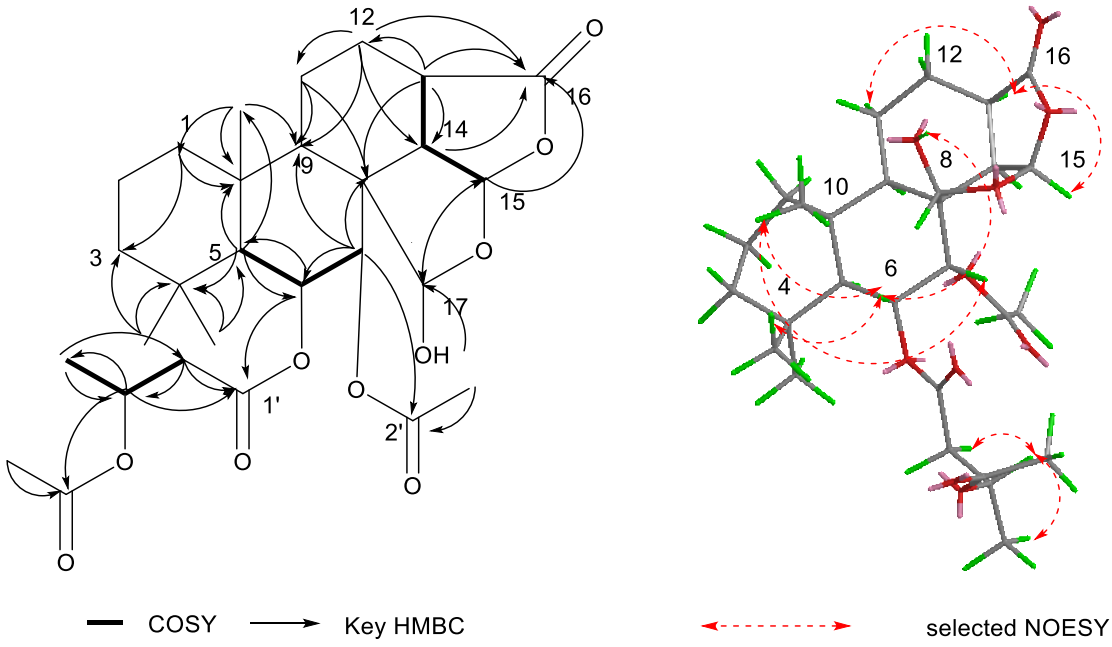


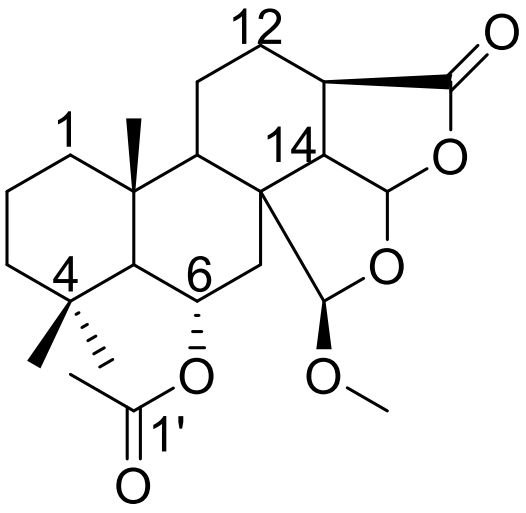
Figure 3.4.5 COSY, key HMBC and selected NOE correlations of compound 5.

Table 3.4.5 NMR data for compound 5

Position	^{13}C (150 MHz) $CDCl_3$	^{1}H (600 MHz) *J* in Hz
1a	38.8 (t)	0.95, m
1b		1.73, m
2a	18.4 (t)	1.46, m
2b		1.57, m
3a	43.3 (t)	1.23, m
3b		1.37, m
4	32.7 (s)	
5	50.8 (d)	1.73, d, (12.2)
6	71.6 (d)	5.39, dd, (12.2, 2.5)
7	73.5 (d)	4.95, d, (2.5)
8	51.2 (s)	
9	48.8 (d)	1.46, m
10	39.5 (s)	
11a	16.3 (t)	1.44, m
11b		2.02, m
12a	23.2 (t)	1.53, m
12b		2.33, m
13	37.5 (d)	2.72, dd, (11.4, 6.6)
14	42.5 (d)	2.68, dd, (11.4, 5.3)
15	104.5 (d)	5.99, d, (5.3)
16	177.1 (s)	
17	103.4 (d)	5.58, br. s
18	35.8 (q)	0.97, s
19	21.7 (q)	0.86, s
20	16.6 (q)	1.03, s
7-$OCOCH_3$	170.7 (s)	
7-$OCOCH_3$	21.0 (q)	2.17, s
$OCOCH_2CHCH_3$	170.3 (s)	
$OCOCH_2CHCH_3$	41.1 (t)	2.49, d, (6.3)
$OCOCH_2CHCH_3$	67.5 (d)	5.23, m, (6.3)
$OCOCH_2CHCH_3$	19.6 (q)	1.27, d, (6.3)
$OCOCH_3$	170.2 (s)	
$OCOCH_3$	21.1 (q)	1.96, s
17-OH		4.50, s
MeOH	50.6 (q)	3.45, s

3.4.6 Compound 6 (new)

Compound 6：aplyroseol-23	
Biological Source	*Aplysilla rosea*
Sample Code	3K8-D
Sample Amount	2.1 mg
Molecular Formula	$C_{23}H_{34}O_6$
Molecular Weight	406 g/mol
Solubility	MeOH
Physical Description	Colourless solid



Compound **6**, a new compound, was isolated as a colourless solid. The molecular formula was established as $C_{23}H_{34}O_6$ with 7 degrees of unsaturation on the basis of its HRESIMS spectrum, indicating the presence of 7 degrees of unsaturation. All 23 carbons were well resolved in the ^{13}C NMR spectrum, and were classified and correlated protons by the NMR data as three methyl groups (δ_H 0.99, s, 0.92, s, 0.89, s; δ_C 36.3, 22.4, 14.6), a lactone carbon (δ_C 178.2), an ester carbon (δ_C 170.0), an acetate methyl (δ_H 2.02; δ_C 22.1 q), two acetal groups (δ_H 5.97, d, and 4.96, br s; δ_C 102.4 d, and 106.4 d), and a methoxy group (δ_H 3.51; δ_C 57.8 q). These structural features were similar to compound **3**, except the 17-OH in compound **2** was replaced by a methoxy group in compound **6**, which was supported by the HMBC correlations of H-17 to methoxy carbon (δ_C 57.8 s) and H_3-OMe to C-17 (δ_C 106.4 s) (Figure 3.4.6.1).

Briefly, the NMR data of compound **6** showed evidence for four sets of coupled protons at H-5 (δ_H 1.24, d, J = 11.1 Hz) and H-6 (δ_H 5.50, td, J = 11.1, 4.2 Hz), H-6 (δ_H 5.50, td, J = 11.1, 4.2 Hz) and H-7b (δ_H 2.53, dd, J = 12.0, 4.2 Hz), H-13 (δ_H 2.60, dt, J = 10.3, 4.8 Hz) and H-14 (δ_H 2.72, dd, J = 10.3, 5.5 Hz), and H-14 (δ_H 2.72, dd, J = 10.3, 5.5 Hz) and H-15 (δ_H 5.97, d, J = 5.5 Hz) (Table 3.4.6).

The HMBC correlations of H-6/C-1', H-17/C-OMe, and H_3-OMe/C-17, indicated that the acetal ester and methoxy groups attached C-6 and C-17, respectively. The NOESY correlations of H-5/H_3-18, and H_3-20, H-6/H-7_b, H_3-18, and H_3-20, H-15/H-7_b, and H_3-OMe/H_3-20 suggested the three methyls and protons were positioned on the same face, while the NOESY correlations from H-7b to H-14 and H-3b indicated the three protons were positioned on the other face.

The key HMBS, COSY and selected NOESY correlations showed in Figure 3.4.6.2 confirmed this proposal structure. Followed the aplyroseol series, compound **6** was named as aplyroseol-23.

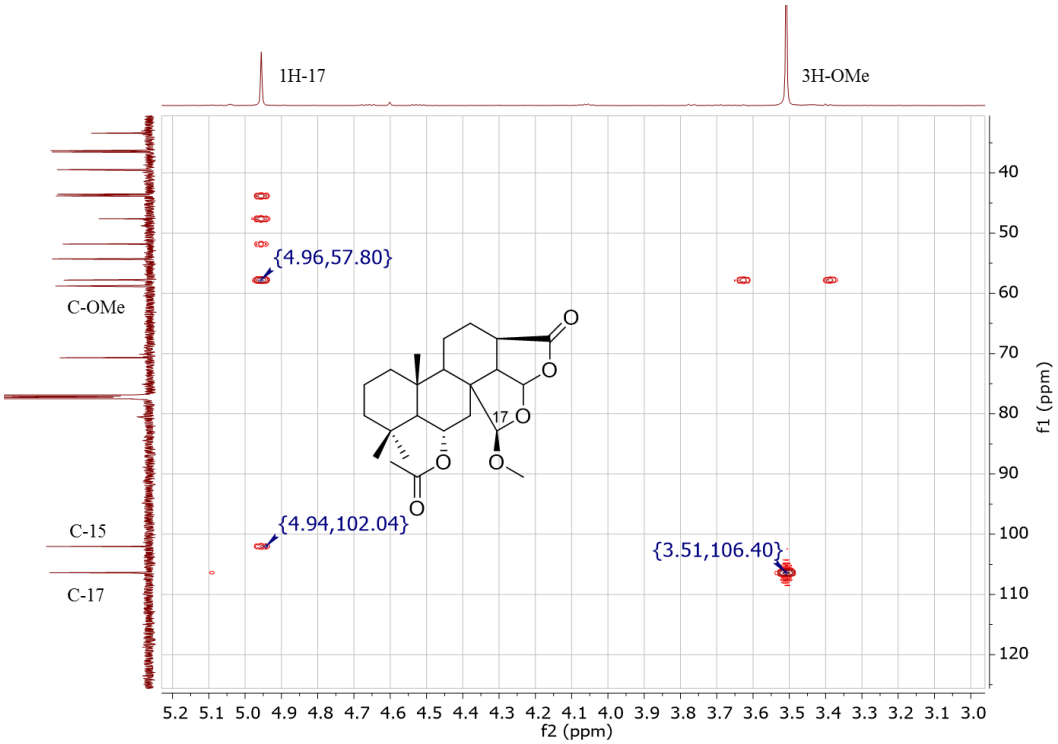


Figure 3.4.6.1 HMBC correlation of compound 6.

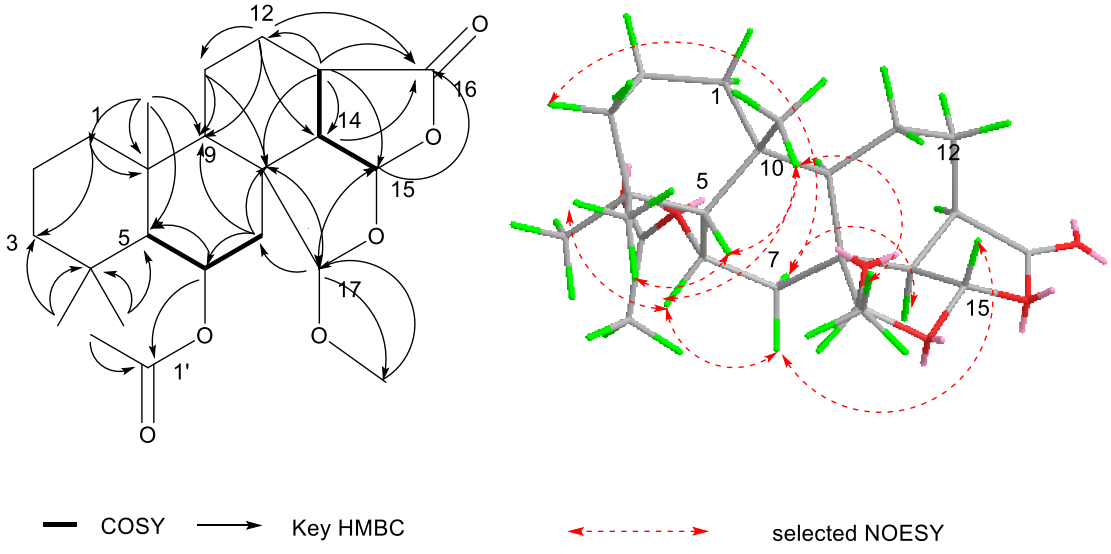


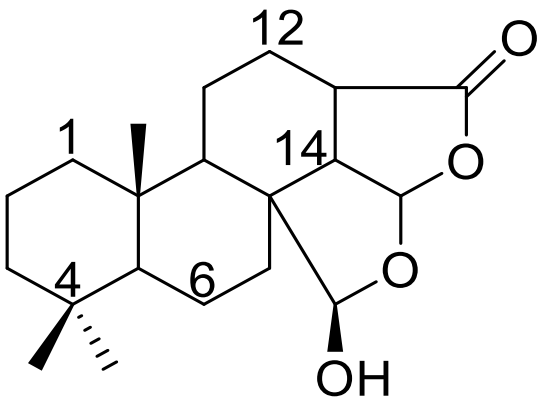
Figure 3.4.6.2. COSY, key HMBC and selected NOE correlations of compound 6.

Table 3.4.6 NMR data for compound 6

Position	^{13}C (150 MHz) $CDCl_3$	^{1}H (600 MHz) *J* in Hz
1a	39.5 (t)	0.93, m
1b		1.64, m
2a	18.5 (t)	1.45, m
2b		1.58, m
3a	43.5 (t)	1.22, m
3b		1.36, m
4	33.4 (s)	
5	58.8 (d)	1.24, d, (11.1)
6	70.7 (d)	5.50, td, (11.1, 4.2)
7a	43.8 (t)	1.14 t, (12.0)
7b		2.53, dd, (12.0, 4.2)
8	47.6 (s)	
9	51.83 (d)	1.36, m
10	39.5 (s)	
11a	17.6 (t)	1.37, m
11b		1.73, m
12a	24.6 (t)	1.92, m
12b		2.00, m
13	36.5 (d)	2.60, dt, (10.3, 4.8)
14	54.3 (d)	2.72, dd, (10.3, 5.5)
15	102.4 (d)	5.97, d, (5.5)
16	178.2 (s)	
17	106.4 (d)	4.96, br. s
18	36.3 (q)	0.99, s
19	22.4 (q)	0.89, s
20	14.6 (q)	0.92, s
$OCOCH_3$	170.0 (s)	
$OCOCH_3$	22.1 (q)	2.02, s
OMe	57.8 (q)	3.51, s

3.4.7 Compound 7 (known)

Compound 7: Dendrillol-1	
Biological Source	*Aplysilla rosea*
Sample Code	3L-1
Sample Amount	200 mg
Molecular Formula	$C_{20}H_{30}O_4$
Molecular Weight	334 g/mol
Solubility	MeOH
Physical Description	Colourless crystal



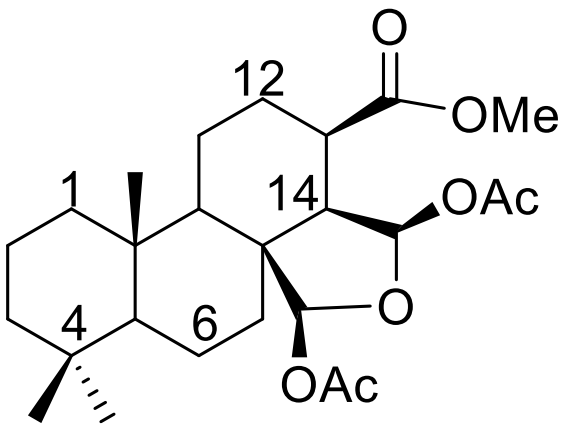
Compound **7** was obtained as a colourless crystal by reverse-phase HPLC. Its molecular weight was indicated as 334 g/mol based on the ESIMS (*m/z* 433.22 $[M + H]^+$) and given the molecular formula as $C_{20}H_{30}O_4$ with 6 degrees of unsaturation. The ^1H and ^{13}C NMR spectra of compound **7** showed evidence for three methyl groups at δ_H (0.82, 3H; 0.84, 3H and 0.91, 3H) and δ_C (16.1, q; 21.5, q and 33.4, q), an γ-lactone at δ_C 177.4 (s), an acetal carbon at δ_C 104.4 (d), a hemiacetal carbon at δ_C 103.9 (d). These structural features were similar to compound **3**, except there was one ester signal disappear (Appendix AR71, AR72 and AR73). These data were found to be the same as those reported for Dendrillol-1 (Table 3.4.7), and compound **7** was consequently identified as Dendrillol-1 (Karuso et al., 1986, Ksebati and Schmitz, 1987, Suciati et al., 2011).

Table 3.4.7 NMR data for compound 7

Position	$CDCl_3$, δ (ppm)	Reference, δ (ppm)
	^{13}C (150 MHz) $CDCl_3$	^{13}C (75 MHz) $CDCl_3$ (Ksebati and Schmitz, 1987)
1	39.1 (t)	39.1 (t)
2	18.8 (t)	18.9 (t)
3	42.0 (t)	41.5 (t)
4	33.3 (s)	33.3 (s)
5	49.6 (d)	49.6 (d)
6	20.1 (t)	20.1 (t)
7	41.5 (t)	41.9 (t)
8	46.9 (s)	46.9 (s)
9	37.8 (d)	37.8 (d)
10	38.1 (s)	38.1 (s)
11	16.6 (t)	16.6 (t)
12	23.9 (t)	23.9 (t)
13	55.5 (d)	55.5 (d)
14	56.8 (d)	56.8 (d)
15	104.4 (d)	104.4 (d)
16	177.4 (s)	177.4 (s)
17	103.9 (d)	103.9 (d)
18	33.4 (q)	33.4 (q)
19	21.5 (q)	21.4 (q)
20	16.1 (q)	16.1 (q)

3.4.8 Compound 8 (known)

Compound 8: 6*H*-Phenanthro[1,10a-*c*]furan-3-carboxylic acid, 4,6-bis(acetyloxy)tetradecahydro-9,9,12a-trimethyl-, methyl ester	
Biological Source	*Aplysilla rosea*
Sample Code	3S4-C3
Sample Amount	23.0 mg
Molecular Formula	$C_{25}H_{38}O_7$
Molecular Weight	450 g/mol
Solubility	MeOH
Physical Description	Colourless oil



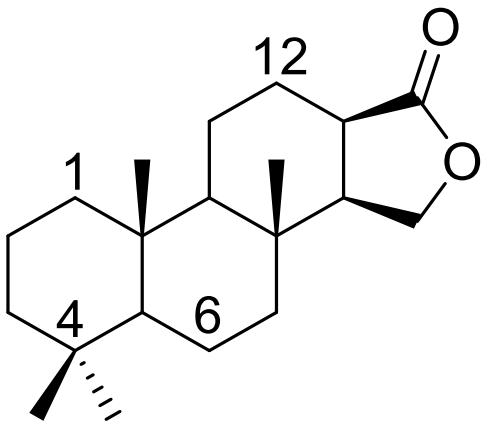
Compound **8** was isolated as colourless oil. The molecular formula of $C_{25}H_{38}O_7$ with 7 degrees of unsaturation was deduced from its ESIMS data (*m/z* 451.5 $[M + H]^+$) in combination with extensive NMR analyses. The NMR data for compound **8** indicated six methyl groups (δ_H 0.77, s, 0.77, s, 0.85, s, 2.03, s, 2.11, s, 3.67, s), a lactone (δ_C 174.3), two esters (δ_C 169.9, 169.4), two acetal carbons (δ_C 99.6, 99.3), and a methyl ester (δ_C 51.9) (Appendix AR81, AR82 and AR83). The esters identified as acetate by the presence of two three-proton singlet at 2.03 and 2.11 ppm plus ^{13}C signals at 21.2 (q)/21.3 (q) and 169.9 (s)/169.4 (s) ppm. Therefore, compound **8** was finally confirmed as 6*H*-Phenanthro[1,10a-*c*]furan-3-carboxylic acid, 4,6-bis(acetyloxy)tetradecahydro-9,9,12a-trimethyl-, methyl ester by analysis of all data as mentioned above together with comparing the data with reported values (Table 3.4.8) (Ksebati and Schmitz, 1987).

Table 5.4.8 NMR data for compound 8

Position	$CDCl_3$, δ (ppm)	Reference, δ (ppm)
	^{13}C (150 MHz) $CDCl_3$	^{13}C (75 MHz) $CDCl_3$ (Ksebati and Schmitz, 1987)
1	37.9 (t)	37.9 (t)
2	18.3 (t)	18.4 (t)
3	41.8 (t)	41.8 (t)
4	33.0 (s)	33.1 (s)
5	56.2 (d)	56.3 (d)
6	20.3 (t)	20.4 (t)
7	38.9 (t)	38.9 (t)
8	47.5 (s)	47.6 (s)
9	49.2 (d)	49.2 (d)
10	37.9 (s)	38.0 (s)
11	15.8 (t)	15.8 (t)
12	18.8 (t)	18.8 (t)
13	37.7 (d)	37.7 (d)
14	56.8 (d)	56.9 (d)
15	99.6 (d)	99.6 (d)
16	174.3 (s)	174.4 (s)
17	99.3 (d)	99.2 (d)
18	33.5 (q)	33.5 (q)
19	21.5 (q)	21.6 (q)
20	14.0 (q)	14.0 (q)
$OCOCH_3$	169.4 (s)	169.4 (s)
$OCOCH_3$	169.9 (s)	169.9 (s)
$OCOCH_3$	21.2 (q)	21.2 (q)
$OCOCH_3$	21.3 (q)	21.3 (q)
OMe	51.9 (q)	51.9 (q)

3.4.9 Compound 9 (known)

Compound 9: Spongian-16-one	
Biological Source	*Aplysilla rosea*
Sample Code	3T2-D
Sample Amount	12.5 mg
Molecular Formula	$C_{20}H_{32}O_2$
Molecular Weight	304 g/mol
Solubility	MeOH
Physical Description	Colourless solid



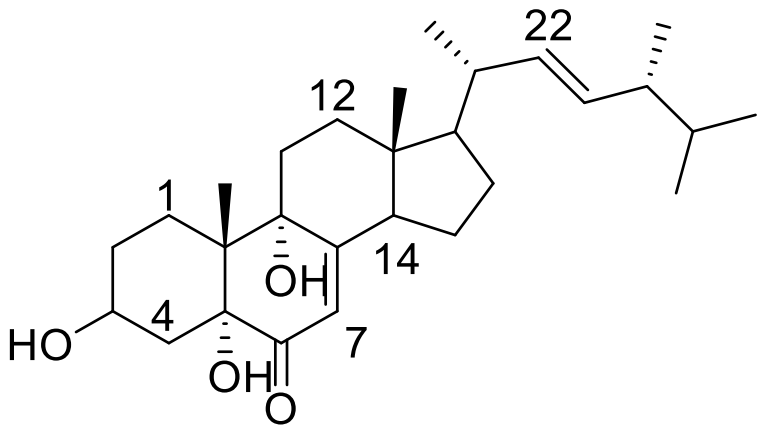
Compound **9** was obtained as colourless solid. The molecular formula was established as $C_{20}H_{32}O_2$ with 5 degrees of unsaturation on the basis of its ESIMS spectrum. All 20 carbons were well resolved in the 13C NMR spectrum, and were classified by chemical shift, DEPT spectrum as four quaternary carbons (δ_C 179.2, 37.5, 35.9, and 33.5), four methyl groups (δ_C 33.5, 21.7, 16.5, and 15.6), and a lactone (δ_C 179.2) (Appendix AR91 and AR92). The ^{1}H spectrum of compound **9** showed two sets of coupled protons at H-15α (δ_H 4.09, dd, *J* = 9.7, 5.4 Hz), H-15β (δ_H 4.20, d, *J* = 9.7 Hz), and H-14 (δ_H 2.08, dd, *J* = 7.7, 5.4 Hz) (Appendix AR91). Based on these data and a comparison with data available in the literature (Table 3.4.9), compound **9** was established to be Spongian-16-one (Kernan et al., 1990).

Table 3.4.9 NMR data for compound 9

Position	$CDCl_3$, δ (ppm)	Reference, δ (ppm)
	^{13}C (150 MHz) $CDCl_3$	^{13}C (75 MHz) $CDCl_3$ (Kernan et al., 1990)
1	40.2 (t)	40.0 (t)
2	18.7 (t)	18.5 (t)
3	42.1 (t)	41.9 (t)
4	33.5 (s)	33.3 (s)
5	56.8 (d)	56.7 (d)
6	18.1 (t)	17.9 (t)
7	42.3 (t)	42.1 (t)
8	35.9 (s)	35.7 (s)
9	56.5 (d)	56.4 (d)
10	37.5 (s)	37.3 (s)
11	17.4 (t)	17.2 (t)
12	22.5 (t)	22.4 (t)
13	37.6 (d)	37.4 (d)
14	50.7 (d)	50.5 (d)
15	67.7 (t)	67.6 (t)
16	179.2 (s)	179.0 (s)
17	15.6 (q)	15.5 (q)
18	33.5 (q)	33.3 (q)
19	21.7 (q)	21.5 (q)
20	16.5 (q)	16.3 (q)

3.4.10 Compound 10 (known)

Compound 10 : 3*β*,5*α*,9*α*-trihydroxy-(22E,24R)-ergosta-7,22-dien-6-one	
Biological Source	*Aplysilla rosea*
Sample Code	3T8-A
Sample Amount	5.2 mg
Molecular Formula	$C_{28}H_{44}O_4$
Molecular Weight	444 g/mol
Solubility	MeOH
Physical Description	Colourless needles



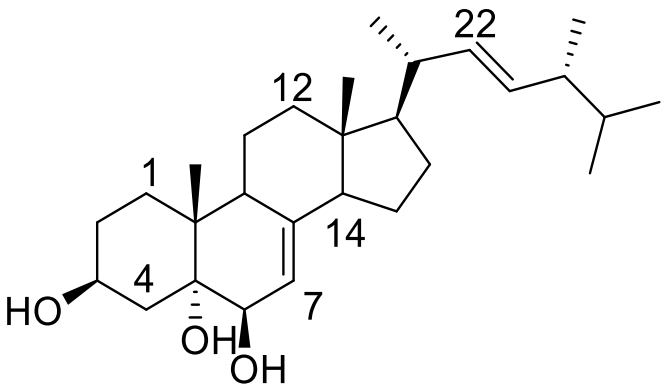
Compound **10** was purified as colourless needles of molecular formula $C_{28}H_{44}O_4$ with 7 degrees of unsaturation by ESIMS at *m/z* 443.4 $[M - H]^-$. The ^1H NMR spectrum of **10** showed the presence of signals characteristic of sterols at δ0.61 (s, 3H), 1.01 (s, 3H), 1.03 (d, *J* = 6.60 Hz, 3H), 0.83 (d, *J* = 6.85, 8.25 Hz, 3H), 0.83 (d, *J* = 6.85, 8.25 Hz, 3H) and 0.92 (d, J = 6.80 Hz, 3H) (Appendix AR101), corresponding to six different methyl groups as showed in ^{13}C NMR data. The ^{13}C NMR also observed the presence of a carbonyl group at δ 197.5, and three oxygenated carbon signals at δ 79.6, 74.7 and 67.2 (Appendix AR102 and AR103). These data were found to be the same as those reported for 3*β*,5α,9α-trihydroxy-(22E,24R)-ergosta-7,22-dien-6-one (Table 3.4.10), and compound **10** was consequently identified as 3*β*,5*α*,9*α*-trihydroxy-(22E,24R)-ergosta-7,22-dien-6-one (Yaoita et al., 2001, Wang et al., 2014).

Table 3.4.10 NMR data for compound 10

Position	C_5D_5N, δ (ppm)	Reference, δ (ppm)
	^{13}C (150 MHz) $CDCl_3$	^{13}C (125 MHz) $CDCl_3$ (Wang et al., 2014)
1	25.6 (t)	25.4 (t)
2	30.3 (t)	30.0 (t)
3	67.4 (d)	67.2 (d)
4	37.5 (t)	37.1 (t)
5	74.9 (s)	74.7 (s)
6	197.5 (s)	197.7 (s)
7	120.1 (d)	119.8 (d)
8	164.2 (s)	164.4 (s)
9	79.9 (s)	79.6 (s)
10	43.2 (s)	42.4 (s)
11	29.0 (t)	28.8 (t)
12	35.0 (t)	34.9 (t)
13	45.5 (s)	45.3 (s)
14	51.9 (d)	51.7 (d)
15	22.6 (t)	22.4 (t)
16	28.3 (t)	27.8 (t)
17	56.1 (d)	56.0 (d)
18	12.3 (q)	12.2 (q)
19	20.7 (q)	20.4 (q)
20	40.5 (d)	40.2 (d)
21	21.3 (q)	21.0 (q)
22	135.4 (d)	135.0 (d)
23	132.7 (d)	132.5 (d)
24	42.0 (d)	42.8 (d)
24 (1)	18.2 (q)	17.6 (q)
25	33.3 (d)	33.0 (d)
26	19.8 (q)	19.6 (q)
27	20.3 (q)	19.9 (q)

3.4.11 Compound 11 (known)

Compound 11 : 24-Methylcholesta-7,22-diene-3β,5α,6β-triol	
Biological Source	*Aplysilla rosea*
Sample Code	3T9-B
Sample Amount	15.1 mg
Molecular Formula	$C_{28}H_{46}O_3$
Molecular Weight	430 g/mol
Solubility	MeOH
Physical Description	Colourless needles



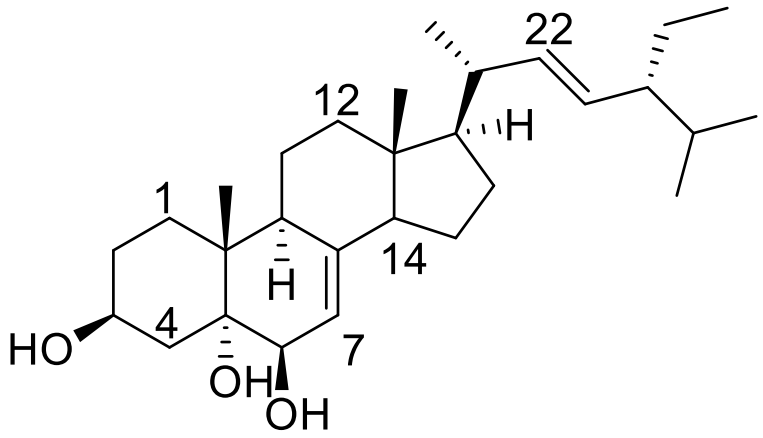
Compound **11** was obtained as colourless needles with a molecular formula $C_{28}H_{46}O_3$ with 6 degrees of unsaturation by ESIMS (*m/z* 430 $[M]^+$). The ^{13}C NMR spectrum of **11** confirmed the presence of three hydroxyl groups with signals at 76.0 (s), 74.1 (t) and 67.5 (t) ppm (Appendix AR112 and AR113). The ^1H NMR spectrum of compound **11** indicated the presence six methyl groups at δ 0.65 (3H, s, H-18), 0.80 (3H, d, *J* = 1.8 Hz, H-27), 0.85 (3H, d, *J* = 1.8 Hz, H-26), 0.94 (3H, d, *J* = 6.8 Hz, H-24(1)), 1.04 (3H, d, *J* = 6.6 Hz, H-21) and 1.53 (3H, s, H-19). Also, two one-proton signals at δ 4.34 (1H, br s, H-6α) and 4.84 (1H, m, H-3α) were assigned to the hydroxymethine protons (Appendix AR111). Based on these data and a comparison with data available in the literature (Table 3.4.11), compound **11** was determined to be 24-Methylcholesta-7,22-diene-3β,5α,6β-triol (Iorizzi et al., 1988, Hata et al., 2002b, Lee et al., 2011).

Table 3.4.11 NMR data for compound 11

Position	C_5D_5N, δ (ppm)	Reference, δ (ppm)
	^{13}C (150 MHz) C_6D_5N	^{13}C (63 MHz) C_6D_5N (Lee et al., 2011)
1	33.8 (t)	33.7 (t)
2	32.6 (t)	32.5 (t)
3	67.6 (d)	67.5 (d)
4	42.0 (t)	41.8 (t)
5	76.1 (s)	76.0 (s)
6	74.2 (d)	74.1 (d)
7	120.5 (d)	120.4 (d)
8	141.5 (s)	141.4 (s)
9	43.7 (d)	43.6 (d)
10	38.1 (s)	37.9 (s)
11	22.4 (t)	22.3 (t)
12	39.9 (t)	39.8 (t)
13	43.7 (s)	43.6 (s)
14	55.2 (d)	55.1 (d)
15	23.5 (t)	23.3 (t)
16	28.7 (t)	28.4 (t)
17	56.1 (d)	56.0 (d)
18	12.5 (q)	12.4 (q)
19	18.8 (q)	18.7 (q)
20	40.9 (d)	40.8 (d)
21	21.4 (q)	21.3 (q)
22	136.4 (d)	136.1 (d)
23	132.2 (d)	132.0 (d)
24	43.3 (d)	42.9 (d)
25	33.5 (d)	33.2 (d)
26	19.8 (q)	19.7 (q)
27	20.3 (q)	20.0 (q)
28	18.2 (q)	17.7 (q)

3.4.12 Compound 12 (known)

Compound 12: 24-Ethylcholesta-7,22-diene-3β,5α,6β-triol	
Biological Source	*Aplysilla rosea*
Sample Code	3T9-C
Sample Amount	3.0 mg
Molecular Formula	$C_{29}H_{48}O_3$
Molecular Weight	444 g/mol
Solubility	MeOH
Physical Description	Colourless needles



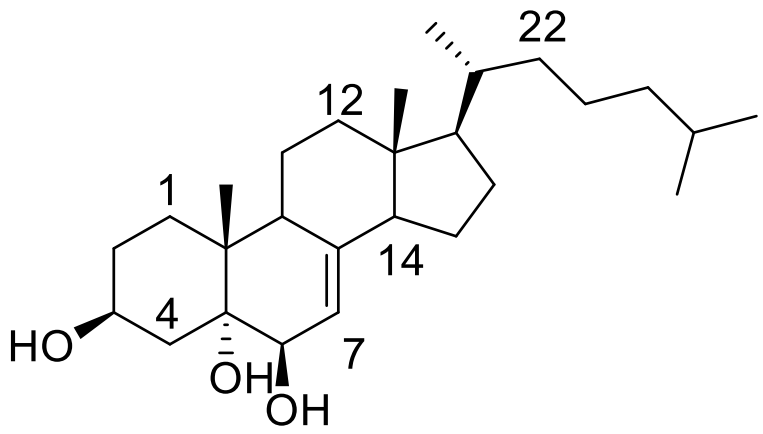
Compound **12** was purified as a colourless needles that gave a $[M]^+$ ion at *m/z* 444 in the ESIMS, matching the molecular formula $C_{29}H_{48}O_3$. The ^1H NMR spectrum of compound **12** showed the presence six methyl groups at δ 0.65 (3H, s, H-18), 0.82 (3H, d, *J* = 1.6 Hz, H-27), 0.85 (3H, d, *J* = 1.6 Hz, H-26), 0.94 (3H, d, *J* = 6.8 Hz, H-24(2)), 1.05 (3H, d, *J* = 6.6 Hz, H-21) and 1.52 (3H, s, H-19). Two oxygenated methane protons (H-3, 6) were appeared at δ 4.31 (1H, br s, H-6) and 4.82 (1H, m, H-3) (Appendix AP121). The ^{13}C NMR spectrum of **12** indicated the presence of three hydroxyl groups' singlets at 76.1 (s), 74.3 (d) and 67.6 (d) ppm. The presence of six methyl groups' signals was confirmed at 22.4 (q), 20.1 (q), 19.8 (q), 18.8 (q), 17.8 (q) and 12.5 (q) ppm (Appendix AR122 and AR123). These data were found to be the same as those reported for 24-Ethlycholesta-7,22-diene-3β,5α,6β-triol (Table 3.4.12), and compound **12** was consequently identified as 24-Ethylcholesta-7,22-diene-3β,5α,6β-triol (Lee et al., 2011, Tian et al., 2014).

Table 3.4.12 NMR data for compound 12

Position	C_5D_5N, δ (ppm)	Reference, δ (ppm)
	^{13}C (150 MHz) C_6D_5N	^{13}C (75 MHz) C_6D_5N (Lee et al., 2011)
1	32.6 (t)	31.9 (t)
2	33.3 (t)	32.6 (t)
3	67.6 (d)	66.9 (d)
4	42.0 (t)	41.3 (t)
5	76.1 (s)	75.4 (s)
6	74.2 (d)	73.5 (d)
7	120.5 (d)	119.8 (d)
8	141.5 (s)	140.8 (s)
9	43.7 (d)	43.0 (d)
10	38.1 (s)	37.3 (s)
11	22.4 (t)	21.7 (t)
12	39.9 (t)	39.1 (t)
13	43.7 (s)	43.0 (s)
14	55.2 (d)	54.5 (d)
15	23.5 (t)	22.7 (t)
16	28.5 (t)	27.8 (t)
17	56.1 (d)	55.3 (d)
18	12.5 (q)	11.8 (q)
19	18.8 (q)	18.1 (q)
20	40.8 (d)	40.2 (d)
21	20.1 (q)	19.4 (q)
22	136.2 (d)	135.5 (d)
23	132.1 (d)	131.1 (d)
24	43.0 (d)	42.3 (d)
25	33.8 (d)	33.1 (d)
26	21.4 (q)	20.7 (q)
27	19.8 (q)	19.1 (q)
28	30.0 (t)	29.3 (t)
29	17.8(q)	17.1 (q)

3.4.13 Compound 13 (known)

Compound 13: 5α-chlest-7-ene-3β,5,6β-triol	
Biological Source	*Aplysilla rosea*
Sample Code	3T9-D
Sample Amount	2.0 mg
Molecular Formula	$C_{27}H_{46}O_3$
Molecular Weight	418 g/mol
Solubility	MeOH
Physical Description	Colourless needles

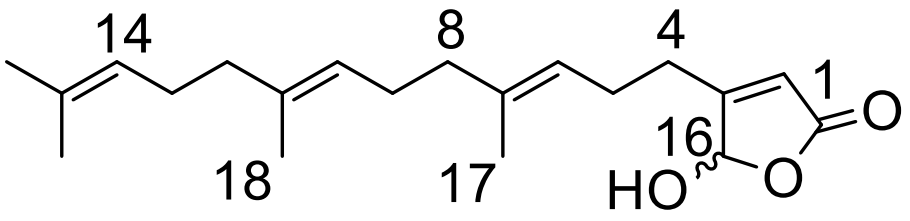


Compound **13**, obtained as a colourless, needle after RP-HPLC, and gave the molecular formula $C_{27}H_{46}O_3$ by ESIMS. The ions at 287 $[M\text{-}C_8H_{17}]^+$ and 251 $[M\text{-}C_8H_{17}\text{-}3H_2O]^+$ indicated the presence of a C_8H_{17} side chain. The ^{13}C NMR spectrum of **13** showed the presence of three hydroxyl groups with signals at δ 76.1 (s), 74.2 (d) and 67.6 (d). The presence of five methyl groups' signals was indicated at δ 22.9 (q), 22.7 (q), 19.0 (q), 18.8 (q), and 12.5 (q) (Appendix AR132 and AR133). The ^1H NMR spectrum of compound **13** confirmed the presence five methyl groups at δ 0.63 (3H, s, H-18), 0.85 (3H, d, *J* = 6.6 Hz, H-27), 0.86 (3H, d, *J* = 6.6 Hz, H-26), 0.95 (3H, d, *J* = 6.4 Hz, H-21) and 1.52 (3H, s, H-19) (Appendix AR131). These data were found to be the same as those reported for 5α-chlest-7-ene-3β,5,6β-triol (Table 3.4.13), and compound **13** was consequently identified as 5α-chlest-7-ene-3β,5,6β-triol (Piccialli and Sica, 1987, Migliuolo et al., 1991).

Table 3.4.13 NMR data for compound 13

Position	C_5D_5N, δ (ppm)	Reference, δ (ppm)
	^{13}C (150 MHz) C_6D_5N	^{13}C (67.9 MHz) C_6D_5N (Migliuolo et al., 1991)
1	32.6 (t)	32.3 (t)
2	33.8 (t)	32.6 (t)
3	67.6 (d)	67.2 (d)
4	42.0 (t)	41.0 (t)
5	76.1 (s)	75.9 (s)
6	74.2 (d)	73.5 (d)
7	120.4 (d)	120.2 (d)
8	141.6 (s)	141.1 (s)
9	43.7 (d)	43.7 (d)
10	38.0 (s)	39.2 (s)
11	22.4 (t)	21.7 (t)
12	40.0 (t)	40.0 (t)
13	43.8 (s)	43.9 (s)
14	55.1 (d)	55.1 (d)
15	23.5 (t)	23.2 (t)
16	28.2 (t)	28.3 (t)
17	56.5 (d)	56.4 (d)
18	12.3 (q)	12.3 (q)
19	18.8 (q)	17.9 (q)
20	36.4 (d)	36.5 (d)
21	19.0 (q)	19.1 (q)
22	36.5 (t)	36.6 (t)
23	24.3 (t)	24.4 (t)
24	39.7 (t)	39.8 (t)
25	28.2 (t)	28.3 (t)
26	22.9 (q)	23.0 (q)
27	22.7 (q)	22.8 (q)

3.4.14 Compound 14 (known)

Compound 14: 2(5*H*)-Furanone,5-hydroxy-4-[(3*E*,7*E*)-4,8,12-trimethyl-3,7,11-tridecatrien-1-yl]	
Biological Source	*Aplysilla rosea*
Sample Code	3U4-A
Sample Amount	11.2 mg
Molecular Formula	$C_{20}H_{30}O_3$
Molecular Weight	318 g/mol
Solubility	MeOH
Physical Description	Light-red oil
	

Compound **14** was obtained as light-red oil. The molecular was established as $C_{20}H_{30}O_3$ on the basis of its ESIMS spectrum, indicating the presence of 6 degrees of unsaturation. All 20 carbons were well resolved in the ^{13}C NMR spectrum, and were classified by chemical shifts, DEPT and HSQC spectra as five quaternary carbons (δ_C 171.6, 169.5, 137.6, 135.5, and 131.5), four olefinic methines (δ_C 124.5, 124.0, 122.1, and 117.8), a hemiacetal carbon (δ_C 99.2) (Appendix AR141-AR147).

The COSY cross-peaks of H-5/H-6, H-8/H-9, H-9/H-10, H-12/H-13, and H-13/H-14 suggested that connectivity of C_5-C_6, C_8-C_9-C_{10}, and C_{12}-C_{13}-C_{14}, respectively. The HMBC correlations of H_3-17/C-7, H_3-18/C-11, H_3-19/C-15, and C-20, and H_3-20/C-15 indicated the four methyls attached C-7, C-11, and C-15, respectively. The COSY, key HMBC and selected NOESY correlations were showed in Figure 3.4.14. Therefore, compound **14** was finally confirmed as 2(5*H*)-Furanone,5-hydroxy-4-[(3*E*,7*E*)-4,8,12-trimethyl-3,7,11-tridecatrien-1-yl] by analysis of all data as mentioned above together with comparing the available data in the literature (Table 3.4.14) (Walker and Faulkner, 1981, Kernan and Faulkner, 1988, Parsons and Du Bois, 2013, Presley et al., 2015). Compound **14** was the first time isolated from natural source; however, the configuration at C-16 could not be determined.

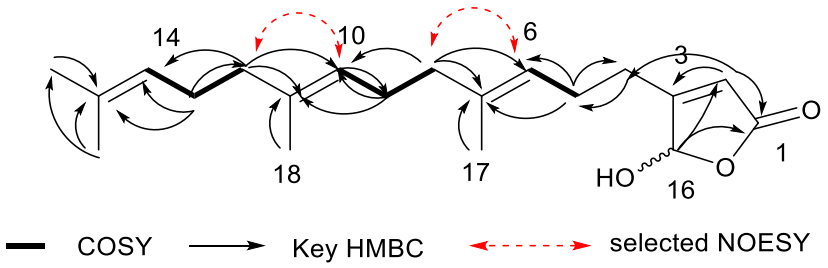


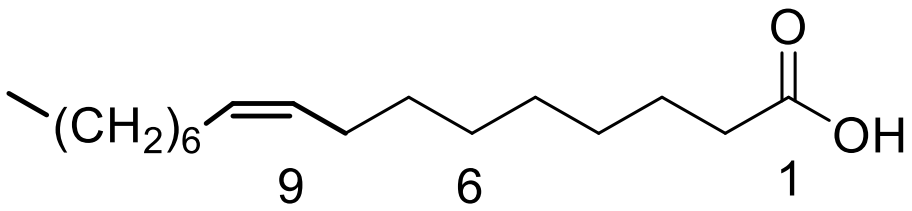
Figure 3.4.14 COSY and key HMBC correlations of compound 14.

Table 3.4.14 NMR data for compound 14

Position	$CDCl_3$, δ (ppm)		Reference, δ (ppm) (Presley et al., 2015)	
	^{13}C (150 MHz) $CDCl_3$	^{1}H (600 MHz)	^{13}C (75 MHz) $CDCl_3$	^{1}H (300 MHz)
1	171.6 (s)		170.7 (s)	
2	117.8 (d)	5.86, s	118.1 (d)	5.84, s
3	169.5 (s)		168.8 (s)	
4	27.9 (t)	2.46, br d	27.9 (t)	
5	25.3 (t)	2.31, m	25.4 (t)	
6	122.1 (d)	5.12, t	122.2 (d)	5.10, t
7	137.6 (s)		137.7 (s)	
8	39.7 (t)	1.97, m	39.8 (t)	
9	26.9 (t)	2.05, m	26.9 (t)	
10	124.0 (d)	5.09, t	124.0 (d)	5.10, t
11	135.5 (s)		135.5 (s)	
12	39.9 (t)	1.97, m	39.9 (t)	
13	26.6 (t)	1.97, m	26.6 (t)	
14	124.5 (d)	5.09, t	124.5 (d)	5.10, t
15	131.5 (s)		131.5 (s)	
16	98.5 (d)	6.00, s	98.5 (d)	6.00, s
17	16.2 (q)	1.59, s	16.2 (q)	
18	16.3 (q)	1.63, s	16.4 (q)	
19	17.8 (q)	1.61, s	17.7 (q)	
20	25.8 (q)	1.68, s	25.9 (q)	

3.4.15 Compound 15 (known)

Compound 15: (*9Z*)-*Heptadec-9-enoic Acid*	
Biological Source	*Aplysilla rosea*
Sample Code	3U4-D
Sample Amount	6.0 mg
Molecular Formula	$C_{17}H_{32}O_2$
Molecular Weight	268 g/mol
Solubility	MeOH
Physical Description	Colourless oil



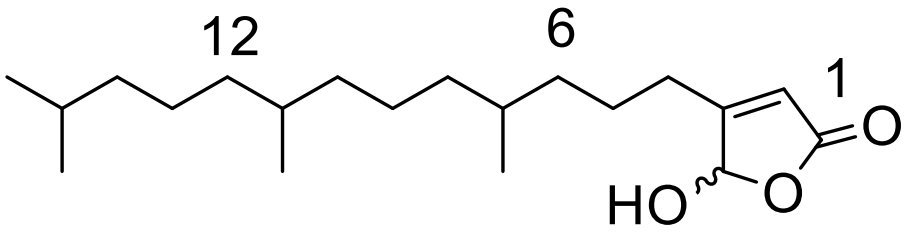
Compound **15**, isolated as colourless oil, had a molecular formula of $C_{17}H_{32}O_2$ with 2 degrees of unsaturation by ESIMS and 1D NMR of compound **15**; as expected for a fit acid, there were a methyl group (δ_H 0.88, 3H), thirteen methylene signals (δ_C 22.9-34.1, t), a C=C bond signal (δ_C 130.0 and 130.1, d) and a ester signal (δ_C 179.7, s) (Appendix AR151, AR152 and AR153). These data were found to be the same as those reported for (*9*Z)-*Heptadec-9-enoic Acid* (Table 3.4.15), and compound **15** was consequently identified as (*9*Z)-*Heptadec-9-enoic Acid* (Bruns et al., 2013).

Table 3.4.15 NMR data for compound 15

Position	1 $CDCl_3$, δ (ppm)	Reference, δ (ppm)
	^{13}C (150MHz), $CDCl_3$	^{13}C (75 MHz), $CDCl_3$ (Bruns et al., 2013)
1	179.7 (s)	179.6 (s)
2	34.1 (t)	33.9 (t)
3	24.8 (t)	24.7 (t)
4	29.4 (t)	29.3 (t)
5	29.2 (t)	29.0 (t)
6	29.2 (t)	29.1 (t)
7	29.4 (t)	29.2 (t)
8	27.4 (t)	27.2 (t)
9	130.1 (d)	130.0 (d)
10	130.0 (d)	129.7 (d)
11	27.4 (t)	27.1 (t)
12	29.6 (t)	29.5 (t)
13	29.9 (t)	29.8 (t)
14	29.6 (t)	29.7 (t)
15	31.9 (t)	31.9 (t)
16	22.8 (t)	22.7 (t)
17	14.3 (q)	14.1 (q)

3.4.16 Compound 16 (known)

Compound 16: Cacospongionolide C	
Biological Source	*Aplysilla rosea*
Sample Code	3U5-B
Sample Amount	22.0 mg
Molecular Formula	$C_{20}H_{26}O_3$
Molecular Weight	324 g/mol
Solubility	MeOH
Physical Description	Colourless oil



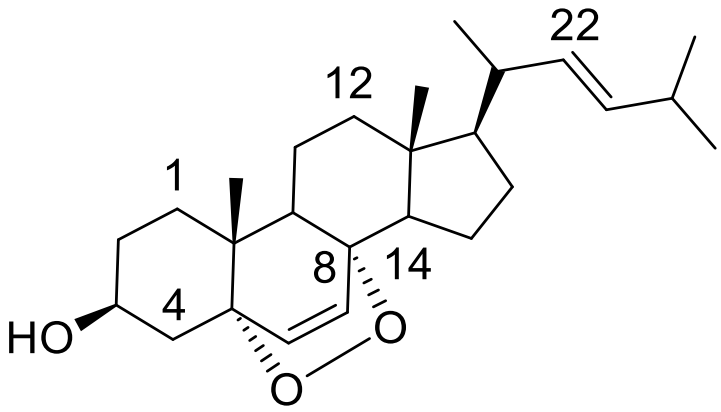
Compound **16** was obtained as colourless oil reversed-phase HPLC, and had a molecular formula of $C_{20}H_{26}O_3$. The ^{1}H and ^{13}C spectrum of compound **16** revealed signals at δ_H 6.01 (br. s, 1H) and 5.83 (br. S, 1H), and δC 172.1 (s), 170.5 (s), 117.3 (d) and 99.4 (d), which were defined the γ-hydroxybutenolide moiety (De Rosa et al., 1988). The presence of four methyl groups' signals was showed at δ_H (0.83, 0.85, 0.86 and 0.87) and δ_C (19.7, 19.9, 22.8 and 22.9). The evidence of a saturated acyclic chain from C-4 to C-19 (δ_C 22.3 to 39.5) was indicated from ^{13}C and DEPT NMR spectrum of compound **16** (Appendix AR161, AR162 and AR163). Based on these data and a comparison with data available in the literature (Table 3.4.16), compound **16** was confirmed to be Cacospongionolide C (De Rosa et al., 1988, De Rosa et al., 1995).

Table 3.4.16 NMR data for compound 16

Position	1 $CDCl_3$, δ (ppm)	Reference, δ (ppm)
	^{13}C (150 MHz), $CDCl_3$	^{13}C (125 MHz), $CDCl_3$ (De Rosa et al., 1995)
1	172.1 (s)	170.8 (s)
2	117.3 (d)	117.6 (d)
3	170.5 (s)	169.3 (s)
4	28.1 (t)	28.0 (t)
5	24.9 (t)	24.8 (t)
6	37.4 (t)	37.3 (t)
7	32.9 (d)	32.8 (d)
8	37.5 (t)	37.4 (t)
9	24.3 (t)	24.2 (t)
10	37.4 (t)	37.3 (t)
11	32.7 (d)	32.6 (d)
12	36.8 (t)	36.6 (t)
13	24.6 (t)	24.4 (t)
14	39.5 (t)	39.4 (t)
15	28.1 (d)	27.9 (d)
16	99.4 (d)	98.6 (d)
17	19.9 (q)	19.7 (q)
18	19.8 (q)	19.6 (q)
19	22.9 (q)	22.7 (q)
20	22.8 (q)	22.6 (q)

3.4.17 Compound 17 (known)

Compound 17: (22*E*)-5α,8α-epidioxy-24-nor-cholesta-6,22-dien-3β-ol	
Biological Source	*Aplysilla rosea*
Sample Code	3U8-A
Sample Amount	2.7 mg
Molecular Formula	$C_{26}H_{40}O_3$
Molecular Weight	400 g/mol
Solubility	MeOH
Physical Description	Colourless oil



Compound **17** was obtained as colourless oil with a molecular formula $C_{26}H_{40}O_3$ by ESIMS (*m/z* 401 $[M + H]^+$). The ^{13}C NMR and DEPT spectrum of compound **17** indicated four olefinic methines (δ_C 135.6, d, 135.5, d, 133.1, d, and 130.9, d), four quaternary carbons (δ_C 82.3, 79.6, 44.7, and 37.1) (Appendix AR172 and AR173). The ^1H NMR spectrum of compound **17** indicated the methyl protons of C-21 (δ_H 0.99, 3H, d, *J* = 6.6 Hz), C-26 (δ_H 0.95, 3H, d, *J* = 6.8 Hz), C-27 (δ_H 0.95, 3H, d, *J* = 6.8 Hz), C-19 (δ_H 0.89, 3H, s), and C-18 (δ_H 0.82, 3H, s) (Figure 3.4.17). Based on these data and a comparison with data available in the literature (Table 3.4.17), this sterol was determined to be (22*E*)-5α,8α-epidioxy-24-nor-cholesta-6,22-dien-3β-ol (Gunatilaka et al., 1981, Ioannou et al., 2009, Mun et al., 2015).

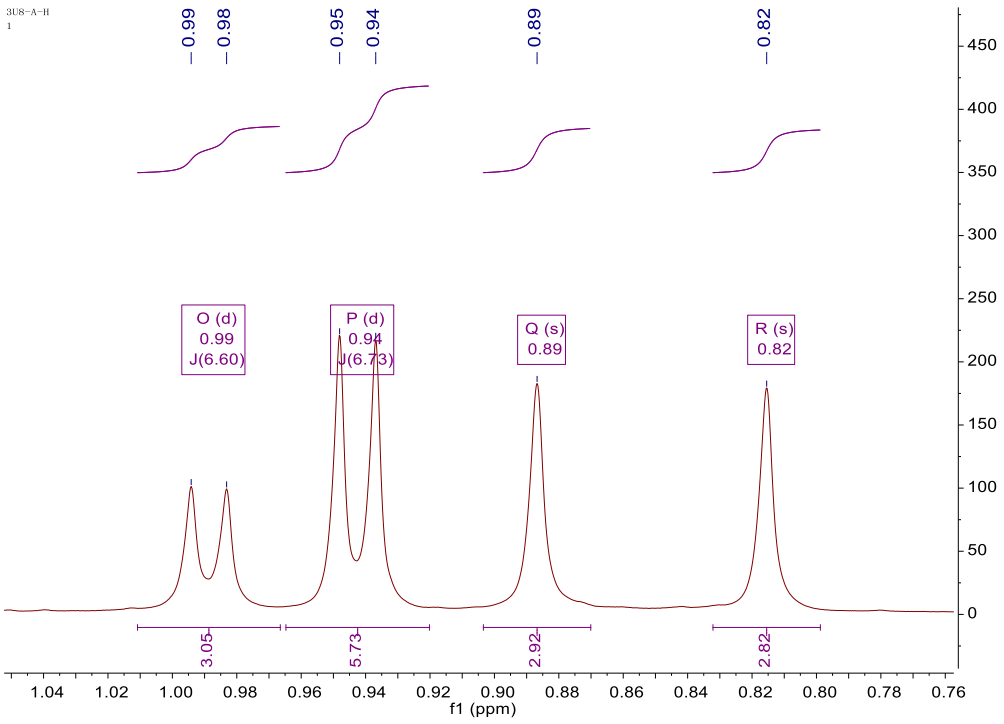


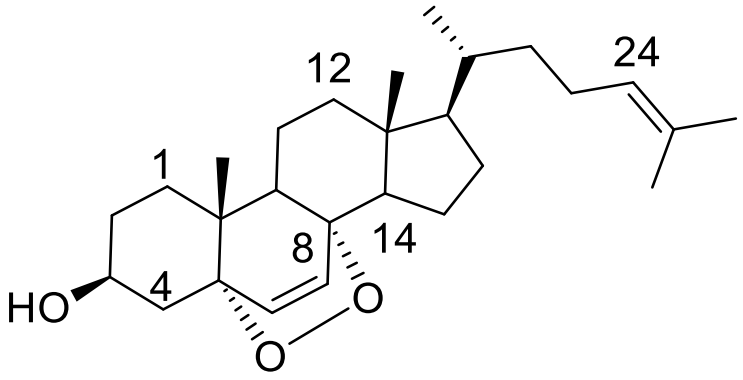
Figure 3.4.17 Selected ^1H NMR date of compound 17.

Table 3.4.17 NMR data for compound 17

Position	$CDCl_3$, δ (ppm), J in Hz		Reference, δ (ppm), J in Hz (Ioannou et al., 2009)	
	^{13}C (150MHz)	^{1}H (600 MHz), $CDCl_3$	^{13}C (75 MHz)	^{1}H (300 MHz), $CDCl_3$
1a	34.8 (t)		34.7 (t)	1.95, m
1b				1.68, m
2a	30.3 (t)		30.2 (t)	1.83, m
2b				1.53, m
3	66.6 (d)	3.97, tt, (10.5, 5)	66.5 (d)	3.95, tt, (10.5, 5.1)
4a	37.1 (t)		37.0 (t)	2.09, m
4b				1.89, m
5	82.3 (s)		82.1 (s)	
6	135.5 (d)	6.24, d, (8.5)	135.4 (d)	6.22, d, (8.5)
7	130.9 (d)	6.51, d, (8.5)	130.7 (d)	6.48, d, (8.5)
8	79.6 (s)		79.4 (s)	
9	51.2 (d)		51.1 (d)	1.48, m
10	37.1 (s)		37.0 (s)	
11a	23.6 (t)		23.4 (t)	1.50, m
11b				1.20, m
12a	39.5 (t)		39.4 (t)	1.93, m
12b				1.21, m
13	44.7 (s)		44.6 (s)	
14	51.8 (d)		51.7 (d)	1.55, m
15a	20.7 (t)		20.6 (t)	1.56, m
15b				1.40, m
16a	28.7 (t)		28.5 (t)	1.73, m
16b				1.31, m
17	56.3 (d)		56.2 (d)	1.18, m
18	13.0 (q)	0.82, s	12.9 (q)	0.79, s
19	18.3 (q)	0.89, s	18.2 (q)	0.86, s
20	39.6 (d)		39.4 (d)	1.98, m
21	20.8 (q)	0.99, d, (6.6)	20.7 (q)	0.96, d, (6.6)
22	133.1 (d)	5.15, dd, (15.3, 8)	133.0 (d)	5.12, dd, (15.3, 8)
23	135.6 (d)	5.28, dd, (15.3, 6)	135.4 (d)	5.27, dd, (15.3, 6)
24				
25	31.1(d)		30.9 (d)	2.16, m
26	22.9 (q)	0.95, d, (6.7)	22.7 (q)	0.92, d, (6.7)
27	22.9 (q)	0.95, d, (6.7)	22.7 (q)	0.92, d, (6.7)

3.4.18 Compound 18 (known)

Compound 18: 5α,8α-epidioxycholesta-6,24-dien-3β-ol	
Biological Source	*Aplysilla rosea*
Sample Code	3U8-B
Sample Amount	2.1 mg
Molecular Formula	$C_{27}H_{42}O_3$
Molecular Weight	414 g/mol
Solubility	MeOH
Physical Description	Colourless oil



Compound **18** was obtained as colourless oil. The molecular formula was established as $C_{27}H_{42}O_3$ on the basis of its ESIMS spectrum, indicating the presence of 7 degrees of unsaturation. The ^{13}C NMR data for compound **18** indicated an overall structure similar to compound **17**. All 27 carbons were well resolved in the ^{13}C NMR spectrum, and were classified by chemical shifts and DEPT spectrum as four olefinic methines (δ_C 135.5, d, 131.3, s, 130.9, d, and 125.1, d), four quaternary carbons (δ_C 82.3, d, 79.6, d, 44.9, d, and 37.1, d) (Appendix AR182 and AR183). The ^1H NMR spectrum of compound **18** indicated the methyl protons of C-26 (δ_H 1.68, 3H, s), C-27 (δ_H 1.60, 3H, s,), C-21 (δ_H 0.93, 3H, d, J = 6.4 Hz), C-19 (δ_H 0.89, 3H, s), and C-18 (δ_H 0.81, 3H, s) (Figure 3.4.18). Compared the NMR data between Compound **17** and **18**, the olefinic carbons C-22, 23 (**17**) were changed to C24, 25 (**18**). Based on these data and a comparison with data available in the literature (Table 3.4.18), this sterol was determined to be 5α,8α-epidioxycholesta-6,24-dien-3β-ol (Jiménez et al., 1986).

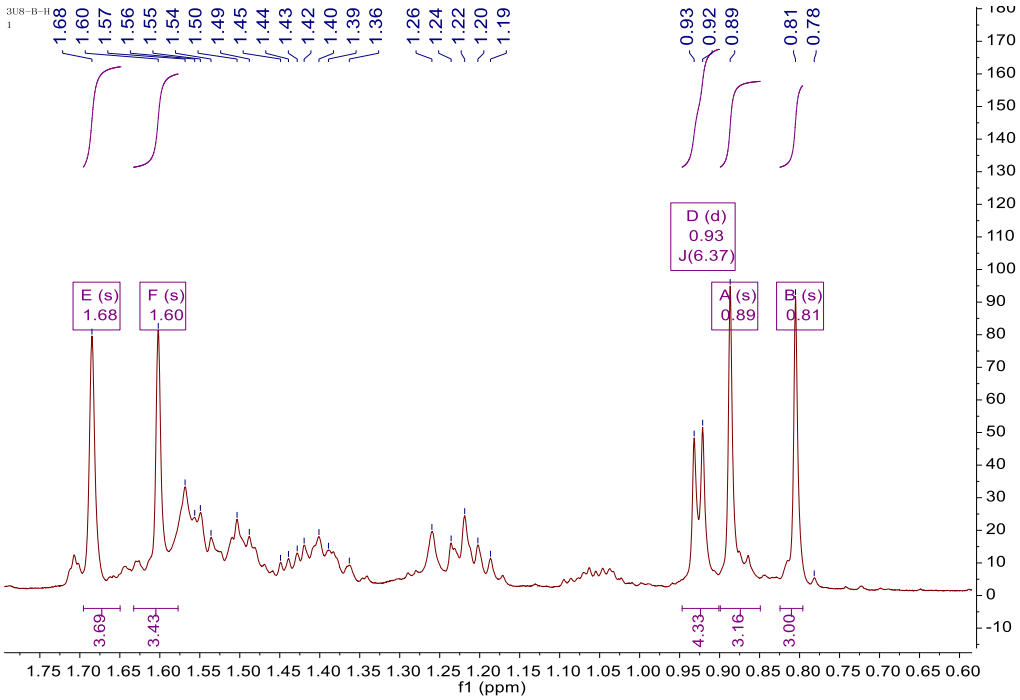


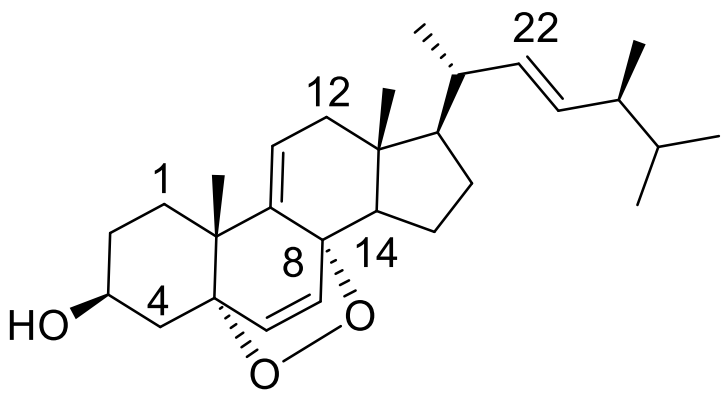
Figure 3.4.18 Selected ^1H NMR date of compound 18

Table 3.4.18 NMR data for compound 18

Position	1 $CDCl_3$, δ (ppm)	Reference, δ (ppm)
	^{13}C (150 MHz), $CDCl_3$	^{13}C (75 MHz), $CDCl_3$ (Jiménez et al., 1986)
1	34.9 (t)	34.7 (t)
2	30.3 (t)	30.1 (t)
3	66.6 (d)	66.5 (d)
4	37.1 (t)	36.9 (t)
5	82.3 (s)	82.2 (s)
6	135.5 (d)	135.5 (d)
7	130.9 (d)	130.8 (d)
8	79.6 (s)	79.4 (s)
9	51.2 (d)	51.2 (d)
10	37.1 (s)	36.9 (s)
11	23.6 (t)	23.4 (t)
12	39.6 (t)	39.5 (t)
13	44.9 (s)	44.8 (s)
14	51.7 (d)	51.6 (d)
15	24.8 (t)	24.7 (t)
16	28.4 (t)	28.2 (t)
17	56.5 (d)	56.4 (d)
18	12.8 (q)	12.6 (q)
19	17.8 (q)	17.6 (q)
20	35.2 (d)	35.0 (d)
21	18.7 (q)	18.5 (q)
22	36.0 (t)	35.8 (t)
23	26.9 (t)	26.5 (t)
24	125.1 (d)	125.0 (d)
25	131.3 (s)	131.0 (s)
26	20.8 (q)	20.6 (q)
27	18.3 (q)	18.1 (q)

3.4.19 Compound 19 (known)

Compound 19: 5α,8α-epidioxy-24(*S*)-methylcholesta-6,9(11),22-trien-3β-ol	
Biological Source	*Aplysilla rosea*
Sample Code	3U8-C
Sample Amount	2.3 mg
Molecular Formula	$C_{28}H_{42}O_3$
Molecular Weight	426 g/mol
Solubility	MeOH
Physical Description	Colourless oil



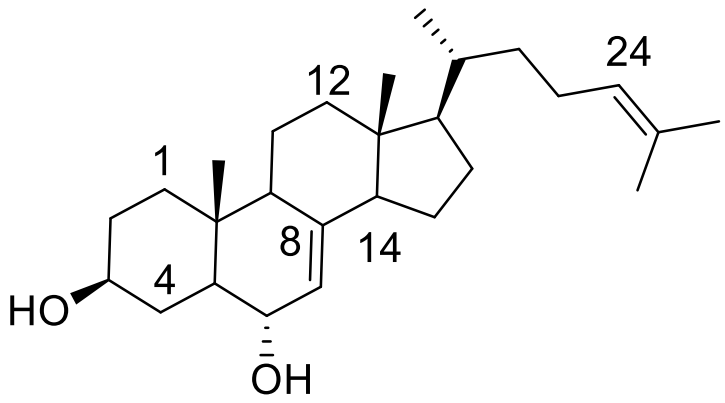
Compound **19** was isolated as a colourless oil. The molecular formula was established as $C_{28}H_{42}O_3$ with 8 degrees of unsaturation on the basis of its ESIMS spectrum, indicating the presence of 8 degrees of unsaturation. The ^{13}C NMR and DEPT spectrum of compound **19** indicated six olefinic methines (δ_C 142.7, s, 135.6, d, 135.5, d, 132.0 d, 130.9, d, and 119.9, d), four quaternary carbons (δ_C 82.8, 78.5, 43.8, and 38.1), and six methyl groups (δ_C 25.7, 20.8, 20.8, 20.7, 18.3, and 13.1) (Appendix AR192 and AR193). The ^1H NMR spectrum confirmed six methyl protons of C-18 (δ_H 0.74, 3H, s), C-19 (δ_H 1.09, 3H, s), C-21 (δ_H 1.00, 3H, d, J = 6.6 Hz), C-26 (δ_H 0.87, 3H, d, J = 6.8 Hz), C-27 (δ_H 0.87, 3H, d, J = 6.8 Hz), and C-28 (δ_H 0.82, 3H, s). In the ^1H NMR spectrum, the presence of three sets of coupled protons signal at H-6 (δ_H 6.29, 1H, d, J = 8.5 Hz) and H-7 (δ_H 6.60, 1H, d, J = 8.5 Hz), H-22 (δ_H 5.20, 1H, dd, J = 15.2 Hz) and H-23 (δ_H 5.28, 1H, dd, J = 15.2 Hz), and H-26 (δ_H 0.87, 3H, d, J = 6.8 Hz) and H-27 (δ_H 0.87, 3H, d, J = 6.8 Hz) (Appendix AR191). Based on these data together and a comparison with data available in the literature (Table 3.4.19), this sterol was determined to be 5α,8α-epidioxy-24(*S*)-methylcholesta-6,9(11),22-trien-3β-ol (Gunatilaka et al., 1981, Ioannou et al., 2009).

Table 3.4.19 NMR data for compound 19

Position	1 $CDCl_3$, δ (ppm)	Reference, δ (ppm)
	^{13}C (150 MHz), $CDCl_3$	^{13}C (75 MHz), $CDCl_3$ (Ioannou et al., 2009)
1	32.7 (t)	32.5 (t)
2	30.8 (t)	30.3 (t)
3	66.5 (d)	66.5 (d)
4	36.2 (t)	35.8 (t)
5	82.8 (s)	82.9 (s)
6	135.6 (d)	135.3 (d)
7	130.9 (d)	130.6 (d)
8	78.5 (s)	78.7 (s)
9	142.7 (s)	142.9 (s)
10	38.1 (s)	37.9 (s)
11	119.9 (d)	119.7 (d)
12	41.3 (t)	41.1 (t)
13	43.8 (s)	43.5 (s)
14	48.3 (d)	47.9 (d)
15	21.1 (t)	20.9 (t)
16	28.9 (t)	28.7 (t)
17	55.9 (d)	55.8 (d)
18	13.1 (q)	12.9 (q)
19	25.7 (q)	25.4 (q)
20	40.0 (d)	39.9 (d)
21	20.8 (q)	20.5 (q)
22	135.5 (d)	135.3 (d)
23	132.0 (d)	132.3 (d)
24	43.4 (d)	43.0 (d)
25	33.9 (d)	33.2 (d)
26	20.7 (q)	19.5 (q)
27	20.8 (q)	20.0 (q)
28	18.3 (q)	17.9 (q)

3.4.20 Compound 20 (known)

Compound 20: 5α-cholest-7,24-diene-3β,6α-diol	
Biological Source	*Aplysilla rosea*
Sample Code	3U11-B
Sample Amount	5.1 mg
Molecular Formula	$C_{27}H_{44}O_2$
Molecular Weight	400 g/mol
Solubility	MeOH
Physical Description	White powder



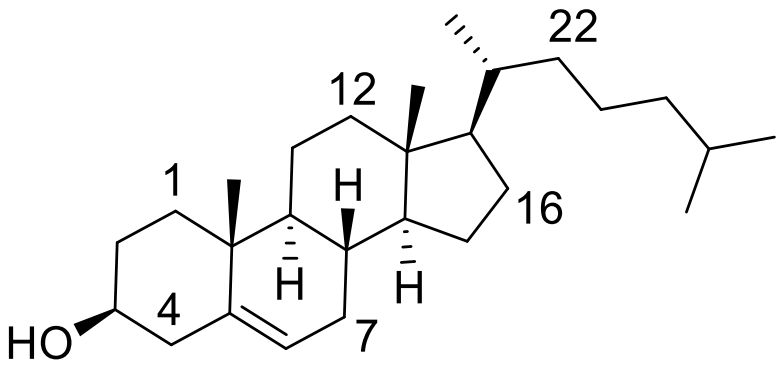
The molecular formula of compound **20** was $C_{27}H_{44}O_2$ by EIMS, with the presence of ion peak at *m/z* 400 $[M]^+$ and 271 $[M^+\text{-}H_2O\text{-}C_8H_{15}]$. The ^1H spectrum of compound **20** indicated five methyl groups at δ 0.54 (3H, s), 0.84 (3H, s), 0.94 (3H, d, *J* = 6.5 Hz), 1.60 (3H, s, H-26 or 27) and 1.58 (3H, s, H-26 or 27). The signals at δ 1.60 (3H, s) and 1.58 (3H, s) have corresponded to the presence of two vinylic methyl groups (Appendix AR201). The ^{13}C and DEPT NMR spectrum of compound **20** showed evidence for two hydroxyl groups with signals at δ 71.0 (s) and 70.2 (s). The five methyl groups' signals were confirmed at δ 25.9 (q), 18.9 (q), 17.8 (q), 14.1 (q) and 12.1 (q) (Appendix AR202 and AR203). Based on these data and a comparison with data available in the literature (Table 3.4.20), compound **20** was established to be 5α-cholest-7,24-diene-3β,6α-diol (Piccialli and Sica, 1986).

Table 3.4.20 NMR data for compound 20

Position	$CDCl_3$, δ (ppm)	Reference, δ (ppm)
	^{13}C (150 MHz), $CDCl_3$	^{13}C (75 MHz), $CDCl_3$ (Piccialli and Sica, 1986)
1	37.3 (t)	37.3 (t)
2	31.2 (t)	31.2 (t)
3	71.0 (d)	70.9 (d)
4	34.0 (t)	33.9 (t)
5	49.0 (d)	49.1 (d)
6	70.2 (d)	70.1 (d)
7	122.0 (d)	122.0 (d)
8	141.8 (s)	141.5 (s)
9	49.3 (d)	49.4 (d)
10	35.3 (s)	35.4 (s)
11	21.5 (t)	21.5 (t)
12	39.5 (t)	39.4 (t)
13	43.8 (s)	43.7 (s)
14	54.9 (d)	54.9 (d)
15	23.0 (t)	22.9 (t)
16	28.1 (t)	27.9 (t)
17	56.2 (d)	56.2 (d)
18	12.1 (q)	11.9 (q)
19	14.1 (q)	13.9 (q)
20	36.1 (d)	35.5 (d)
21	18.9 (q)	18.8 (q)
22	36.1 (t)	36.1 (t)
23	24.9 (t)	24.8 (t)
24	125.3 (d)	125.4 (d)
25	131.2 (s)	130.3 (s)
26	17.8 (q)	17.6 (q)
27	25.9 (q)	25.7 (q)

3.4.21 Compound 21 (known)

Compound 21: 3β-cholest-5-en-3-ol	
Biological Source	*Aplysilla rosea*
Sample Code	3U11-C
Sample Amount	2.0 mg
Molecular Formula	$C_{27}H_{46}O$
Molecular Weight	386 g/mol
Solubility	MeOH
Physical Description	White powder



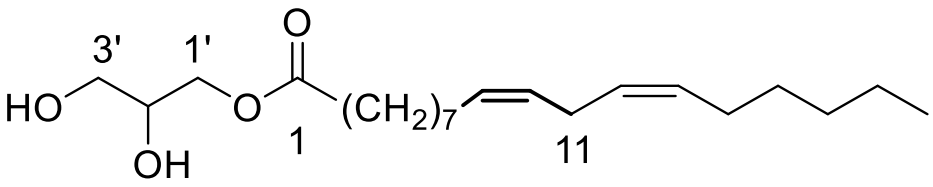
Compound **21** was isolated as white powder with a molecular formula $C_{27}H_{46}O$ by EIMS (*m/z* 386 $[M]^+$). The ^{13}C NMR spectrum of **21** indicated one hydroxyl group with signal at δ 72.0 (d). The evidence of five methyl groups' signal was showed at δ 23.0 (q), 22.7 (q), 19.6 (q), 18.9 (q) and 12.0 (q) (Appendix AR212 and AR213). The ^1H NMR data were closely related to those of compound **13** and **20**, and confirmed five methyl singlets at δ 0.68 (3H, s), 0.86 (3H, d), 0.91 (3H, d), 0.97 (3H, s) and 1.00 (3H, s). The olefinic signal and hydroxyl group were identified at δ 5.35 (1H, br s, H-6), and 3.52 (1H, m, H-3) (Appendix AR211). These data were found to be the same as those reported for 3β-cholest-5-en-3-ol (Table 3.4.21), and compound **21** was consequently identified as 3β-cholest-5-en-3-ol (Rogers and McLaughlin, 1998, Rider et al., 2016).

Table 3.4.21 NMR data for compound 21

Position	$CDCl_3$, δ (ppm)	Reference, δ (ppm)
	^{13}C (150 MHz), $CDCl_3$	^{13}C (75 MHz), $CDCl_3$ (Rogers and McLaughlin, 1998)
1	37.4 (t)	37.3 (t)
2	31.8 (t)	31.9 (t)
3	72.0 (d)	71.7 (d)
4	42.5 (t)	42.3 (t)
5	140.9 (s)	140.8 (s)
6	121.9 (d)	121.6 (d)
7	32.1 (t)	31.9 (t)
8	32.1 (d)	31.8 (d)
9	50.3 (d)	50.2 (d)
10	36.7 (s)	36.5 (s)
11	21.2 (t)	21.1 (t)
12	28.4 (t)	28.2 (t)
13	42.5 (s)	42.3 (s)
14	56.9 (d)	56.8 (d)
15	24.5 (t)	24.3 (t)
16	39.9 (t)	39.8 (t)
17	56.3 (d)	56.2 (d)
18	12.0 (q)	11.9 (q)
19	19.6 (q)	19.4 (q)
20	36.0 (d)	35.8 (d)
21	18.9 (q)	18.7 (q)
22	36.4 (t)	36.2 (t)
23	24.0 (t)	23.8 (t)
24	39.7 (t)	39.5 (t)
25	28.2 (d)	28.0 (d)
26	22.7 (q)	22.6 (q)
27	23.0 (q)	22.8 (q)

3.4.22 Compound 22 (known)

Compound 22: 1-Linoleoylglycerol	
Biological Source	*Aplysilla rosea*
Sample Code	3Y6-A
Sample Amount	9.2 mg
Molecular Formula	$C_{21}H_{38}O_4$
Molecular Weight	354 g/mol
Solubility	MeOH
Physical Description	Colourless oil



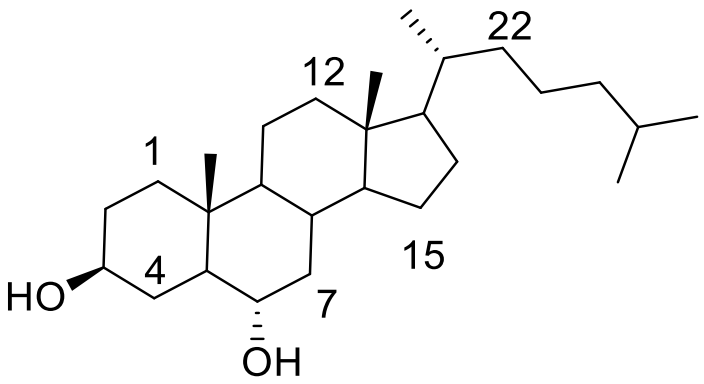
Compound **22** was obtained as colourless oil, and had a molecular formula of $C_{21}H_{38}O_4$. The ^{13}C spectrum of compound **22** indicated one ester singlet at δ 174.3 (s), two C=C bond signals at δ 130.8 (d), 130.8 (d) 129.1 (d) and 128.8 (d), and three hydroxyl group signals at δ 70.4 (d), 65.4 (t) and 63.5 (t) (Appendix AR222 and AR223). The 1H NMR spectrum of compound **22** showed the presence a methyl group at δ 0.88 (3H, t, *J* = 6.9 Hz, H-18), four C=C bond protons (H-9, 10, 12, 13) at δ 5.33, 5.36, 5.40 and 5.43, respectively, and one oxygenated methane proton (H-2′) at 3.94 (1H, m) (Appendix AR221). Based on these data and a comparison with data available in the literature (Table 3.4.22), compound **22** was determined to be 1-Linoleoylglycerol (Murata et al., 2011, Zeng et al., 2012).

Table 3.4.22 NMR data for compound 22

Position	$CDCl_3$, δ (ppm)	Reference, δ (ppm)
	^{13}C (150 MHz), $CDCl_3$	^{13}C (125 MHz), $CDCl_3$ (Kim et al., 2003)
1	174.3 (s)	174.3 (s)
2	33.7 (t)	34.1 (t)
3	25.0 (t)	24.9 (t)
4,	29.5 (t)	29.1 (t)
5,	29.5 (t)	29.2 (t)
6,	29.8 (t)	29.4 (t)
7, 15	29.9 (t)	29.6 (t)
8	27.4 (t)	27.2 (t)
9, 10	130.8 (d)	130.2 (d)
12, 13	129.1 (d)	128.1 (d)
11	26.7 (t)	25.6 (t)
9, 10	128.8 (d)	127.9 (d)
12, 13	130.8 (d)	130.0 (d)
14	27.4 (t)	27.2 (t)
4, 5, 6, 7, 15	29.7 (t)	29.4 (t)
16	32.1 (t)	31.5 (t)
17	22.9 (t)	22.6 (t)
18	14.3 (q)	14.1 (q)
1′	65.4 (t)	65.2 (t)
2′	70.4 (d)	70.3 (d)
3′	63.5 (d)	63.4 (d)

3.4.23 Compound 23 (known)

Compound 23: 5α-cholestane-3β,6α-diol	
Biological Source	*Aplysilla rosea*
Sample Code	3Y7-A
Sample Amount	3.1 mg
Molecular Formula	$C_{27}H_{48}O_2$
Molecular Weight	404 g/mol
Solubility	MeOH
Physical Description	White powder



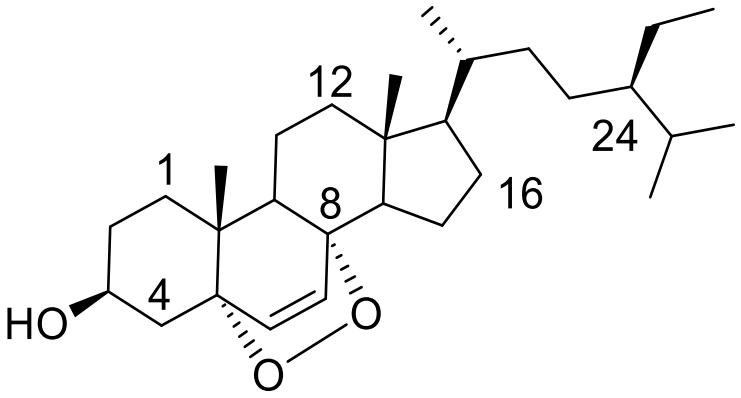
Compound **23** (8.2 mg) was purified as white powder from fraction AR-3Y7 by suing reverse-phase HPLC. The ^{1}H NMR spectrum of compound **23** indicated that it was a steroid. It displayed two singlets of tertiary methyl groups at δ 0.65 (3H, s) and 0.81 (3H, s), a signal at δ 0.89 for the secondary methyl (3H, d, H-21), and two doublet signals at δ 0.85 (3H, d) and 0.86 (3H, d) for the isopropyl group situated in the side chain (Appendix AR231). The presence of two hydroxyl groups was evident from its ^{13}C and DEPT NMR spectrums which displayed signals at δ 71.4 (d) and 69.7 (d). The signals of five methyl groups were also confirmed at δ 23.0 (q), 22.7 (q), 18.8 (q), 13.36 (q) and 12.2 (q) (Appendix AR232 and AR233). Based on these data and a comparison with data available in the literature (Table 3.4.23), compound **23** was confirmed to be 5α-cholestane-3β,6α-diol (Blunt and Stothers, 1977, Wahidulla et al., 1987, Carvalho et al., 2010, Miura et al., 2016).

Table 3.4.23 NMR data for compound 23

Position	$CDCl_3$, δ (ppm)	Reference, δ (ppm)
	^{13}C (150 MHz), $CDCl_3$	^{13}C (75 MHz), $CDCl_3$ (Blunt and Stothers, 1977)
1	37.4 (t)	37.3 (t)
2	31.2 (t)	31.3 (t)
3	71.4 (d)	71.1 (d)
4	32.4 (t)	32.3 (t)
5	51.9 (d)	51.6 (d)
6	69.7 (d)	69.3 (d)
7	41.9 (t)	41.7 (t)
8	34.5 (d)	34.3 (d)
9	54.0 (d)	53.7 (d)
10	36.4 (s)	36.2 (s)
11	21.3 (t)	21.2 (t)
12	40.0 (t)	39.8 (t)
13	42.8 (s)	42.5 (s)
14	56.3 (d)	56.1 (d)
15	24.4 (t)	24.2 (t)
16	28.3 (t)	28.1 (t)
17	56.3 (d)	56.1 (d)
18	12.2 (q)	12.0 (q)
19	13.6 (q)	13.5 (q)
20	35.9 (d)	35.7 (d)
21	18.8 (q)	18.7 (q)
22	36.3 (t)	36.1 (t)
23	24.0 (t)	23.6 (t)
24	39.7 (t)	39.4 (t)
25	28.2 (d)	28.0 (d)
26	22.7 (q)	22.5 (q)
27	23.0 (q)	22.8 (q)

3.4.24 Compound 24 (known)

Compound 24: 5α,8α-epidioxy-24(*R*)-ethylcholesta-6-en-3β-ol	
Biological Source	*Aplysilla rosea*
Sample Code	3Y8-A
Sample Amount	8.2 mg
Molecular Formula	$C_{29}H_{48}O_3$
Molecular Weight	444 g/mol
Solubility	MeOH
Physical Description	Colourless oil



Compound **24** (8.2 mg) was isolated as a colourless oil. The molecular formula was established as $C_{29}H_{48}O_3$ on the basis of its ESIMS spectrum, indicating the presence of 6 degrees of unsaturation. The ^{13}C NMR and DEPT spectrum of compound **24** indicated two olefinic methines (δ_C 135.4, d, and 130.8, d), four quaternary carbons (δ_C 82.2, 79.5, 44.8, and 37.0), and six methyl groups (δ_C 19.9, 18.7, 18.7, 18.2, 12.7, and 12.4) (Appendix AR242 and AR243). In the ^1H NMR spectrum, the presence of six methyl protons of C-18 (δ_H 0.80, 3H, s), C-19 (δ_H 0.88, 3H, s), C-21 (δ_H 0.90, 3H, d, J = 6.5 Hz), C-26 (δ_H 0.82, 3H, d, J = 6.8 Hz), C-27 (δ_H 0.84, 3H, d, J = 6.8 Hz), and C-29 (δ_H 0.85, 3H, t, J = 7.3 Hz), and two sets of coupled protons signal at H-6 (δ_H 6.24, 1H, d, J = 8.5 Hz) and H-7 (δ_H 6.50, 1H, d, J = 8.5 Hz), and H-26 (δ_H 0.82, 3H, d, J = 6.8 Hz) and H-27 (δ_H 0.84, 3H, d, J = 6.8 Hz) (Appendix AR241). Therefore, compound **24** was finally confirmed as 5α,8α-epidioxy-24(*R*)-ethylcholesta-6-en-3β-ol by analysis of all data as mentioned above together with comparing the available data in the literature (Table 3.4.24) (Gauvin et al., 2000, Ioannou et al., 2009).

Table 3.4.24 NMR data for compound 24

Position	$CDCl_3$, δ (ppm)	Reference, δ (ppm)
	^{13}C (150 MHz), $CDCl_3$	^{13}C (75 MHz), $CDCl_3$ (Gauvin et al., 2000)
1	34.8 (t)	34.8 (t)
2	30.2 (t)	30.2 (t)
3	66.5 (d)	66.5 (d)
4	37.0 (t)	37.0 (t)
5	82.2 (s)	82.2 (s)
6	135.4 (d)	135.5 (d)
7	130.8 (d)	130.8 (d)
8	79.5 (s)	79.5 (s)
9	51.1 (d)	51.1 (d)
10	37.0 (s)	37.0 (s)
11	23.5 (t)	23.5 (t)
12	39.5 (t)	39.5 (t)
13	44.8 (s)	44.8 (s)
14	51.6 (d)	51.6 (d)
15	20.7 (t)	20.7 (t)
16	28.3 (t)	28.3 (t)
17	56.3 (d)	56.4 (d)
18	12.7 (q)	12.7 (q)
19	18.2 (q)	18.2 (q)
20	35.8 (d)	36.0 (d)
21	18.7 (q)	18.7 (q)
22	34.8 (t)	34.5 (t)
23	26.1 (t)	26.1 (t)
24	45.8 (d)	45.9 (d)
25	29.2 (d)	29.2 (d)
26	19.9 (q)	19.9 (q)
27	18.7 (q)	18.8 (q)
28	23.1 (t)	23.1 (t)
29	12.4 (q)	12.4 (q)

3.5 Summary

Four new diterpenoids together with 20 known compounds, isolated from sponge *Aplysilla rosea*, were identified in this chapter. Compound 7 (200 mg) is the main secondary metabolite in this sponge. The details of constituents of sponge *Aplysilla rosea* in this study were shown in Table 3.5.

Table 3.5: A summary for constituents of sponge *Aplysilla rosea*

Number			**Number**		
New compound		4	Terpenoid		11
First isolated from natural resource		1	Steroid		11
Known compound		20	Fatty acid		2
Total		24			24
Compound	**Molecular formula**	**Weight /Yield**	**Compound**	**Molecular formula**	**Weight /Yield**
1	$C_{22}H_{32}O_7$	10.6 mg /0.053%	**13**	$C_{27}H_{46}O_3$	2.0 mg /0.010%
2	$C_{26}H_{38}O_9$	8.7 mg/ 0.435%	**14**	$C_{20}H_{30}O_3$	11.2 mg/ 0.056%
3	$C_{22}H_{32}O_6$	23.4 mg /0.117%	**15**	$C_{17}H_{32}O_2$	6.0 mg /0.030%
4	$C_{24}H_{34}O_8$	12.8 mg/ 0.064%	**16**	$C_{20}H_{26}O_3$	22.0 mg/ 0.110%
5	$C_{28}H_{40}O_{10}$	53.0 mg /0.265%	**17**	$C_{26}H_{40}O_3$	2.7 mg /0.014%
6	$C_{23}H_{34}O_6$	2.1 mg/ 0.011%	**18**	$C_{27}H_{42}O_3$	2.1 mg/ 0.011%
7	$C_{20}H_{30}O_4$	200 mg /1.00%	**19**	$C_{28}H_{42}O_3$	2.3 mg /0.012%
8	$C_{25}H_{38}O_7$	23.0 mg/ 0.115%	**20**	$C_{27}H_{44}O_2$	5.1 mg/ 0.026%
9	$C_{20}H_{32}O_2$	12.5 mg /0.063%	**21**	$C_{27}H_{46}O$	2.0 mg /0.010%
10	$C_{28}H_{44}O_4$	5.2 mg/ 0.026%	**22**	$C_{21}H_{38}O_4$	9.2 mg/ 0.046%
11	$C_{28}H_{46}O_3$	15.1 mg /0.076%	**23**	$C_{27}H_{48}O_2$	3.1 mg /0.016%
12	$C_{29}H_{48}O_3$	3.0 mg/ 0.015%	**24**	$C_{29}H_{48}O_3$	8.2 mg/ 0.041%

Chapter 4: Cytotoxicity, Apoptosis Induction and Cell Cycle Arrest of Compounds isolated from *Aplysilla rosea*

4.1 Introduction

The aim of the study presented in this chapter was to evaluate the *in vitro* cytotoxic effect of the compounds obtained from sponge *Aplysilla rosea* on three human breast cancer cell lines (T47D, MCF7 and MDA-MB231) and one normal human breast cell line 184B5. The crystal violet assay was used to detect the number of live cells that are adhered to the plate (dead cells are floating in the treatment and washed away during the assay processing) (Chiba et al., 1998). Cell cycle analysis using PI staining was investigated by flow cytometry. The apoptotic effect of compounds was investigated using Annexin V and PI staining.

4.2 Materials and methods

4.2.1 Materials and reagents

All the reagents used in this chapter were purchased from Sigma-Aldrich unless otherwise noted.

4.2.2 Sample preparation

Twenty-four compounds from *A. rosea* were stored in the -20°C freezer. Samples were dissolved in DMSO at 10 mg/ml and stored at -20 °C until further tests.

4.2.3 Cell culture maintenance

Human adherent breast cancer cells T47D (ductal carcinoma, an endogenously expressing mutant p53 cell line), MCF7 (adenocarcinoma, a p53 wild type cell line)

and MDA-MB-231 (adenocarcinoma, an endogenously expressing mutant p53 cell line) as well as 184B5 cells (human non-cancer breast cell line) were purchased from the American Type Culture Collection. For the description of cell culture maintenance, refer to section **2.2.3** in Chapter two.

4.2.4 Trypan Blue exclusion assay

Trypan Blue exclusion assay was used for cell counting for cell concentration calculation. The details of this assay, was descried in section **2.2.4** in Chapter two.

4.2.5 Cell viability test

Crystal violet assay was used to assess the cytotoxicity effect of compounds from *Aplysilla rosea*. For the detailed description of the method, refer to section **2.2.5** in Chapter two.

4.2.6 Cell cycle analysis

Cell cycle distribution was evaluated by flow cytometry. For the details of this method, refer to section **2.2.6** in Chapter two.

4.2.7 Apoptosis analysis

Apoptosis induction was analysed by flow cytometry using an Annexin V-FITC apoptosis detection kit (BD Biosciences, USA). For the details of this method, refer to section **2.2.7** in Chapter two.

4.2.8 Statistical analysis

Data are presented as the mean ± SEM (standard error of mean). The experiments were replicated at least three independent tests. The IC_{50} calculation was determined using GraphPad V. 6.01 for Windows (GraphPad Software, San Diego, California, USA). Statistical analysis of data was carried out using ANOVA, followed by Tukey's HSD *post hoc* test. All analysis were performed using IBM SPSS Statistics 22 software. A significant difference was considered when the *P*-value was less than 0.05.

4.3 Results

4.3.1 Cytotoxicity of 24 compounds from *Aplysilla rosea*

The cytotoxicity of all 24 compounds was determined by crystal violet assay (IC_{50} value). Compounds **1** – **24** were tested on three human cancer cell lines (T47D, MCF7 and MDA-MB-231) and one non-cancer cell line (184B5) at doses of 1.25, 2.5, 5, 10, and 20 μg/ml for 48 h treatment.

Using the crystal violet assay, cytotoxic effect was observed for compound **1** – **7**, **13**, **16**, **19** and **24** on three cancer cell lines at IC_{50} value range of 3.2 to 16.2 μg/ml. Compound **9** showed cytotoxicity against T47D and MDA-MB-231 cell lines at IC_{50} value of 20.0 and 18.8 μg/ml, respectively. Compound **18** was cytotoxic against T47D cell line, with an IC_{50} value of 19.3 μg/ml. Compound **8**, **10 – 12**, **14**, **15**, **17**, and **20 – 23** did not have any cytotoxicity against all the three breast cancer cell lines (Table 4.1).

In contrast, for the non-cancer cell line, compounds **1**, **3, 5 – 7** caused less cytotoxicity on the non-cancer cell line, as evidenced by significant different ($P < 0.05$) IC_{50} value on 184B5 cell line compared to the IC_{50} values on T47D, MCF7 and MDA-MB-231 cell lines. Especially, the most significant ($P < 0.01$) difference between breast cancer cells and non-breast cancer cells were observed for compound **7** treatment group. Compounds **2**, **13**, **16**, **19** and **24** indicated similar cytotoxicity against non-cancer cell line compared to cancer cell lines (Table 4.1). Considering the results of the cytotoxic effects of compounds **1 – 24** with their yield, compound **7** (200 mg) was the major research object for further cell cycle and apoptosis tests.

Table 4.1 Cytotoxicity (IC_{50}) of 24 compounds from *Aplysilla rosea* estimated by crystal violet assay

Compound	IC50 (at 48 h, μg/ml)			
	T47D	**MCF7**	**MDA-MB-231**	**184B5**
1	9.6 ± 0.5 [a]	8.4 ± 0.3 [a]	10.3 ± 0.1 [a]	15.2 ± 0.4
2	8.7 ± 0.6 [a]	10.3 ± 0.5	11.4 ± 0.3	10.3 ± 0.7
3	6.1 ± 0.8 [a]	6.8 ± 0.8 [a]	7.4 ± 0.5 [a]	15.8 ± 0.4
4	4.6 ± 0.7	3.5 ± 0.2	3.2 ± 0.2	3.8 ± 0.7
5	11.2 ± 0.4 [a]	15.3 ± 0.4 [a]	13.2 ± 0.1 [a]	18.6 ± 0.5
6	9.5 ± 0.6 [a]	8.3 ± 0.5 [a]	8.8 ± 0.4 [a]	11.9 ± 0.6
7	4.4 ± 0.3 [a, b]	3.5 ± 0.1 [a, b]	3.3 ± 0.4 [a, b]	> 20
8	> 20	> 20	> 20	> 20
9	20.0 ± 0.4	> 20	18.8 ± 0.6	> 20
10	> 20	> 20	> 20	> 20
11	> 20	> 20	> 20	> 20
12	> 20	> 20	> 20	> 20
13	12.1 ± 0.2	13.6 ± 0.7	10.6 ± 0.2	11.8 ± 0.4
14	> 20	> 20	> 20	> 20
15	> 20	> 20	> 20	> 20
16	4.3 ± 0.4	5.3 ± 0.5	8.6 ± 0.6 [a]	5.6 ± 0.4
17	> 20	> 20	> 20	> 20
18	19.3 ± 0.6	> 20	> 20	> 20
19	10.6 ± 0.8 [a]	11.5 ± 0.2	14.2 ± 0.2	14.5 ± 0.5
20	> 20	> 20	> 20	> 20
21	> 20	> 20	> 20	> 20
22	> 20	> 20	> 20	> 20
23	> 20	> 20	> 20	> 20
24	13.6 ± 0.8	16.2 ± 0.4	12.5 ± 0.3	13.9 ± 0.4

Note: In 96-well plates for 48 h treatment (at dose of 1.25, 2.5, 5, 10 and 20 μg/ml) against T47D, MCF7, MBA-MB-231, and 184B5. Data are shown as relative surviving cell numbers percentage compare to the medium control (untreated cells, 0 μg/ml) and are mean of three replicates ± S.E.M. The significant different between cancer cell lines and non-cancer cell line showed at $P < 0.05$ as [a], and $P < 0.05$ as [b]. (the IC_{50} of compound 7 on 184B5 was considered as 20 μg/ml).

4.3.2 Effect of Compound 7 on cell cycle arrest in three breast cancer cell lines

Cell cycle distribution was analysed by flow cytometry using PI staining. Compound 7 treatment of three breast cancer cell lines increased the G_0/G_1 peak with a concomitant decrease in the S and G_2/M phases, compared to the control group (concentration = 0 μg/ml) (Figures 4.1). The increase in the G_0/G_1 peak was significant at 10 μg/ml for all three cell lines.

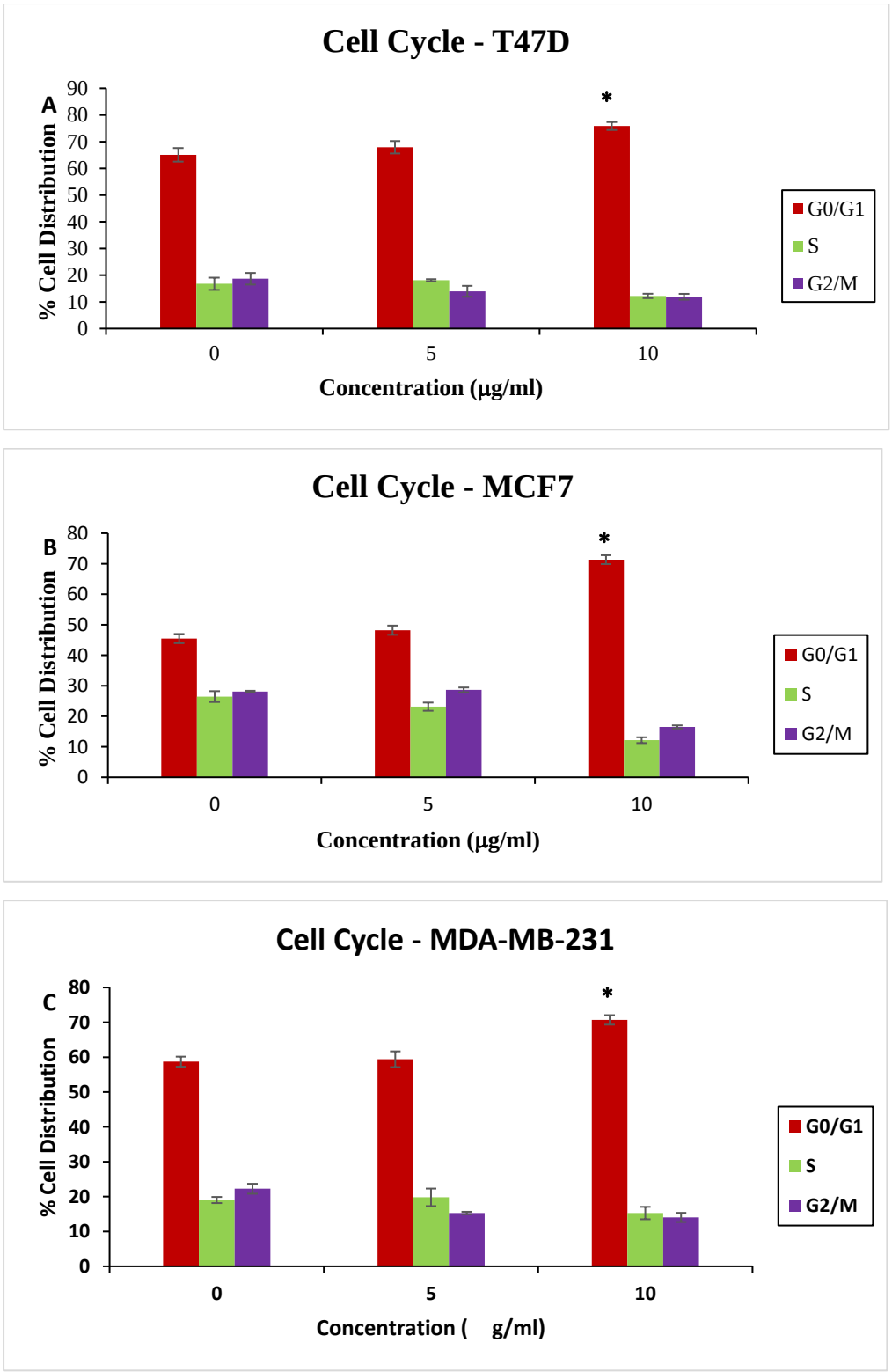


Figure 4.1 Effect of compound 7 (Dendrillol-1) on cell cycle distribution determined by PI staining and analysed for DNA content by flow cytometry

Note: (A) T47D, (B) MCF7, and (C) MDA-MB-231.Data were obtained from 20,000events and are showed as a percentage of cells in the G_0/G_1, S and G_2/M phases. The values are presented as mean ± S.E.M for n = 3. Treatments significantly different from the untreated control at $p < 0.05$ are showed as∗.

4.3.3 4.3.3 Effect of compound 7 on apoptosis induction in three breast cancer cell lines

Treatment with compound 7 induced significant increases in early apoptosis compared to untreated cells in breast cancer cell line T47D (Figure 4.4 A) ($P < 0.01$). The most significant increases were found after treatment with compound 7 at concentration 5 and 10 μg/ml with the percentage of apoptotic cell population at 22 and 33%, respectively, compared to untreated control at 3.3%.

Significant increases were shown after treatment with compound 7 in breast cancer cell line MCF7 (Figure 4.4 B). The most significant increase were observed at concentration of 5 and 10 μg/ml, which induced approximately 20 and 30% early apoptosis, respectively, compared with 3.5% in untreated cells.

Also, significant increases were observed in MDA-MB-231 cell line after treated with compound 7 (Figure 4.4 C). The most significant increase was indicated at higher concentration applied (10 μg/ml) with induced approximately 30% early apoptosis compared with 3.4% in untreated cells.

In the current method, the late apoptosis and necrosis are not distinguishable as cells at both stages are stained by PI and Annexin V-FITC. The significant increases in late apoptosis (or necrosis) were indicated in all three breast cancer cell lines (T47D, MCF7, and MDA-MB-231) after treatment with compound 7 (Figure 4.2 A-C).

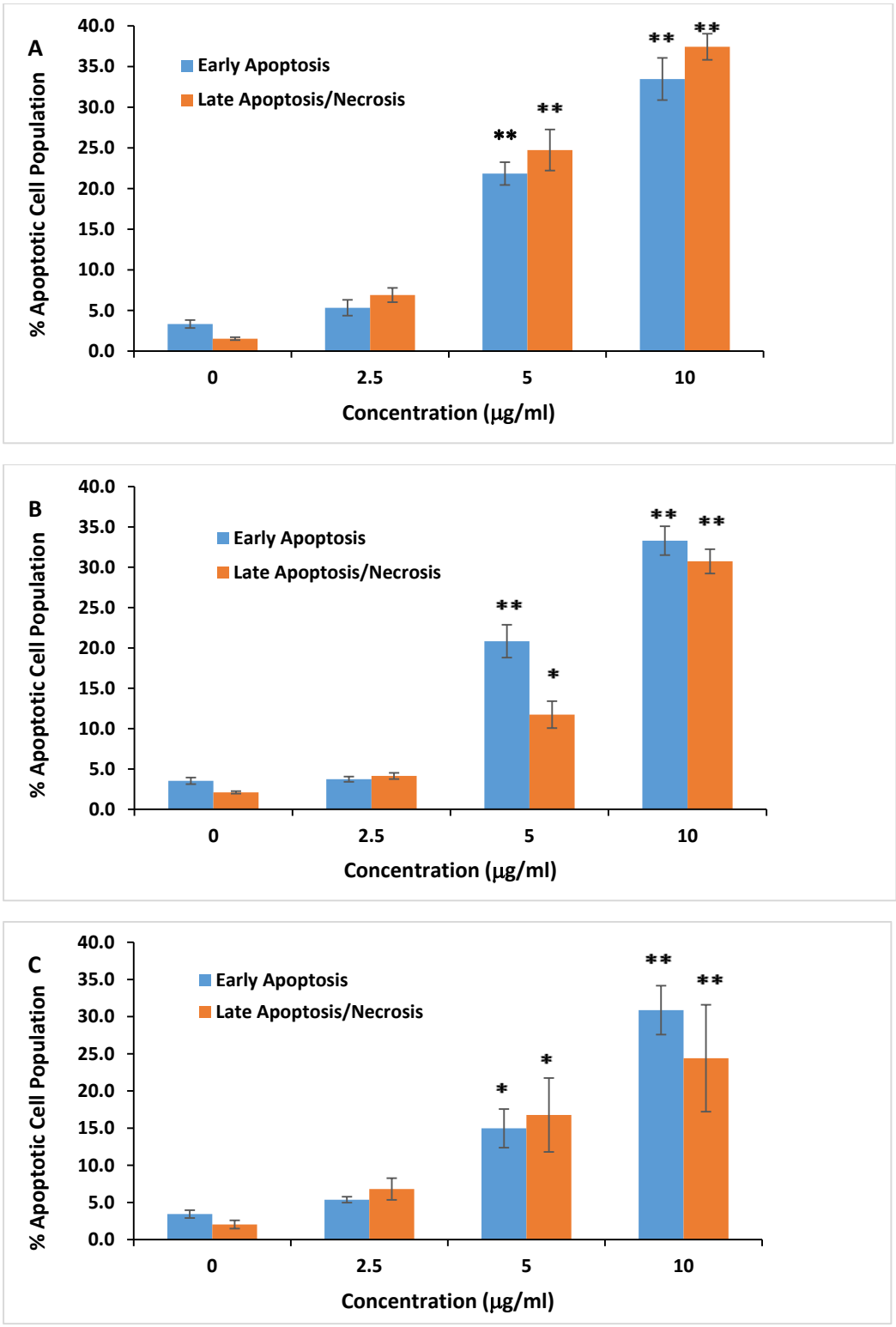


Figure 4.2: Apoptosis effect of compound 7 (Dendrillol-1) on three breast cancer cell lines

Note: (A) T47D, (B) MCF7, and (C) MDA-MB-231.Data were obtained from 20,000 events and early apoptotic cells (Annexin positive) and late apoptotic cell (Annexin positive/PI positive, including necrotic cells) are showed as a percentage of total cells analysed. The values are presented as mean ± S.E.M for n = 3. Treatments significantly different from the untreated control at $p < 0.05$ are showed as∗ and p<0.01 as∗∗.

4.4 Discussion

Aplyroseol series compounds have been isolated from marine sponge *Aplysilla rosea* and some nudibranchs. The compounds from nudibranch may be produced by metabolites obtained from dietary sources, such as sponges (Cimino et al., 1999, Gavagnin and Fontana, 2000, Fontana, 2006).

4.4.1 Cytotoxicity of 24 ested pure compounds

This study is the first demonstration of cytotoxicity induced by these 24 marine sponge *Aplysilla rosea*-derived secondary metabolites on human breast cancer cell lines (except compound **17, 19,** and **24**). The 11 diterpenoids tested exerted growth inhibition against breast cancer cells. Eight of eleven diterpenoids (**1-7, and 16**) obtained from *Aplysilla rosea* showed cytotoxicity with IC_{50} values of 4.3-11.2 μg/ml against T47D cell line, 3.5-15.3 μg/ml against MCF7 cell line, and 3.3-13.2 μg/ml against MDA-MB-231 cell line. Three of eleven steroids (**13, 19, and 24**), isolated in this study, indicated growth inhibition with IC_{50} value of 10.6-13.6 μg/ml for T47D cell line, 11.5-16.2 μg/ml for MCF7, and 10.6-14.2 μg/ml for MDA-MB-231 cell line. The fatty acids (compound **15** and **21**) purified from *Aplysilla rosea* showed no-cytotoxicity against these three human breast cancer cell lines (Table 4.1).

Compound **3**, identified as 6α, 17β-dihydroxy-15, 17-oxidospongian-16-one 6, 17-diacetate, has been isolated from an Australian nudibranch (Ksebati and Schmitz, 1987), but no bioactivity was reported. Compound **3** was found to show anti-proliferation effects on four human cell lines in this study.

Compound **4** is a diterpene and was isolated from sponge *Dendrilla rosea* (Karuso et al., 1986), and no bioactivity reported in the literature. Compound **4** showed a strong cytotoxic effect on three breast cancer cell lines.

Compound **7**, identified as dendrillol-1, has been isolated from sponge *Dendrilla rosea* as compound **4** (Karuso et al., 1986). There was no activity reported for dendrill-1 in the original literature; however, Zaragoza's group indicated that synthetic dendrill-1 induced 100% detachment of Hela (human cervix epithelioid carcinoma) and Hep-2 (human larynx epidermoid carcinoma) at 13 and 13 μg/ml respectively (Betancur-Galvis et al., 2002). Compound **7** was the most toxic compound against three breast cancer cell lines in this study as well.

Compound **8**, **10**, **14**, **15** and **20 – 23** were found to be non-cytotoxic in this study; however, there are no reports on the activities of these compounds in the previous literature (Piccialli and Sica, 1986, Ksebati and Schmitz, 1987, Ioannou et al., 2009, Carvalho et al., 2010, Zeng et al., 2012, Bruns et al., 2013, Parsons and Du Bois, 2013, Wang et al., 2014, Rider et al., 2016).

Compound **9**, spongian-16-one, has been isolated from sponge *Dictyodendrilla cavernosa*, and reported to induced 100% detachment of HeLa and Hep-2 at 20 and 20 μg/ml, respectively as compound **7** (Betancur-Galvis et al., 2002). However, spongian-16-one isolated from Australian nudibranchs was inactive against HeLa S3 cells (Hirayama et al., 2016).

Compound **11** and **12**, steroids, have been isolated from a variety of sponges, mushroom and bacterial (Madaio et al., 1989, Suri et al., 1997, Hata et al., 2002a) and have been report to be candidates as the PTP1B inhibitors (Lee et al., 2011).

Compound **13**, identified as 5α-chlest-7-ene-3β,5,6β-triol, has been purified from gorgonian and sponge, and was found to have activity against A549 (human lung cancer cell line), HT29 (human colon cancer cell line), and MG63 (human bone cancer cell line) with an IC_{50} = 12.2 μM, ED_{50} = 0.23 μM, and IC_{50} = 7.7 μM, respectively (Rueda et al., 2001, Liu et al., 2013).

Compound **16**, Cacospongionolide C, a diterpene has been isolated from sponge *Fasciospongia cavernosa*, and no activity has been reported in the literature. In this study, compound **16** induce strong cytotoxicity against all four human cell lines.

Compound **17**, **19** and **24**, steroids, have been obtained from gorgonian *Eunicella cavolini* and ascidian *Tridemnum inarmatum*. The reports showed growth inhibitory effects on human breast cancer cell line MCF7 with 23%, 11%, and 12% compared to vehicle control at 10 μM (Ioannou et al., 2008, Ioannou et al., 2009).

Compound **18** was identified as 5α,8α-epidioxycholesta-6,24-dien-3β-ol. This steroid was isolated from marine sponge *Monanchora* sp, and had a potent cytotoxic effect against the human colon cancer cell line (HCT 116) with an IC_{50} value of 2.5 μM, and a weak toxic effects on A-498 (human renal cancer cell line), MIA PaCa-2 and PANC-1 (human pancreatic cancer cell line) cells (Mun et al., 2015).

It is noteworthy that five diterpenoids (**1, 3,** and **5-7**) tested in this study displayed selectivity to different extents against human breast cancer cells as evidenced by higher IC_{50} values against normal breast cells (Table 4.1). These data indicate that these diterpenoids tested would have less toxicity on normal breast cells under the conditions tested. These results match the evidence found in Chapter 2. This is a very interested finding, as current anti-cancer drugs have side-effect due to their strong toxicity toward normal cells (Li et al., 2009).

4.4.2 Cell cycle modulatory and apoptotic effect of compound 7 (Dendrillol-1) in human breast cancer cell lines

One of the strategies for cancer therapy is to control cell cycle progression of cancer cells, and it became one of the hallmarks of many common malignancies (Senderowicz, 2003, Molinari, 2000, Pavletich, 1999). Treatment of three breast cancer cell lines (T47D, MCF7 and MDA-MB-231) with compound 7 induced the accumulation of G_0/G_1 phase population with a concomitant decrease in the S and G_2/M phases, indicating induction of apoptosis. Apoptosis plays a major role in defence against cancer (Campbell et al., 2007). There are two main pathways lead to apoptosis, the death receptor pathway (extrinsic) and the mitochondrial pathway (intrinsic). The two pathways are triggered by the binding of death-inducing ligands to cell surface receptors and cytotoxic stresses, respectively (Kettleworth, 2007, Elmore, 2007). A further study to investigate pathway related to apoptosis of compound **7** should be carried out in the future.

Chapter 5: Conclusion & Future Directions

5.1 Conclusions

Australia has the highest breast cancer incidence rate (1/8, before the age of 85) in the female. The resistance of breast cancer to current therapies (chemotherapy, hormone therapy and targeted therapy) call for better treatments for breast cancer patients. Many marine natural products, especially those derived from marine sponges, have demonstrated significant anti-breast cancer activities, which are considered as very important sources to screen for new generation of anti-breast cancer drugs.

The aim of this study was to understand the potential of discovering new anti-breast cancer compounds from untapped South Australian marine sponges, with a specific focus on sponge *Aplysilla rosea*, demonstrated potent anti-breast cancer activities from initial screening of 30 marine sponges. The chemical constituents of *Aplysilla rosea* were focused with a detailed study of isolation and structurally elucidation of chemical sonstituents. Four new compounds together with 20 known compounds were purified from *Aplysilla rosea*, and their structures were elucidated by analysis of spectroscopic data including HRESIMS, and 2D NMR, and confirmed by single-crystal X-ray diffraction analysis. Among these compounds, 22 compounds (including 18 known compounds) were reported for the first time from sponge *Aplysilla rosea*. 50% of those compounds showed cytotoxicity against three breast cancer cells, and 5 compounds indicated selective cytotoxicity against breast cancer cells over on normal breast cells line (184B5). With highest cytotoxicity and selectivity, compound **7** (dendrillol-1) can inhibit cell proliferation of three breast cancer cell lines via cell cycle arrest at G_0/G_1 phase, and apoptosis induction at 10 μg/ml.

Overall, the results from this thesis clearly demonstrated the potential of discovering potent anti-breast cancer compounds from South Australian marine sponges that worth of further in-depth study.

5.2Further directions

5.2.1 Expand the cytotoxicity database of South Australian marine sponges

A preliminary database was established in the Centre for Marine Bioproducts Development (Flinders University), by screening the cytotoxicity of South Australian marine sponges. In the current study, only 30 sponge species were chosen in primary screening; however, there are over 1000 marine sponge species in the South Australian water. In the future, other sponge species could be evaluated on their cytotoxicity against breast cancer cells.

Additionally, three breast cancer cell lines were chosen for this study, as they are the most common breast cancer cell lines for cytotoxicity study of natural products. However they only, represent ductal carcinoma and adenocarcinoma. More studies using a broader range of breast cancer cell lines with different subtypes or genetic backgrounds can be carried out in the future. For example, these different subtypes and genetic backgrounds cell lines could be one in the subtype of luminal B (e.g. BT474), one in the subtype of basal with HER2-positive (e.g. MDA-MB-435), or one with ER-, PR-, and HER2-positive (e.g. MDA-MB-361).

5.2.2 Isolation and purification of other sponge species

The current study chose sponge *Aplysilla rosea* as a target for the study of chemical constituents study as it performed better in screening studies in Chapter 2. We have preliminarily isolated another 20 compounds that need further identification. Therefore there are more new compounds to be discovered from this species. It would be expected that many other species would generate similar scale of new compounds discovered. In further study, sponge *Tedania cf. anhelans* and *Chondropsis* sp. are the good resource for potential anti-cancer compounds discovery. The isolation and purification could be carried out with these sponges and then expanded into other species.

5.2.3 Further comprehensive study of the chemical constituents of *Aplysilla rosea*

In the previous study, there were totally 22 compounds isolated from sponge *Aplysilla rosea*, and they are either diterpenes or lactones. In this study, another 24 compounds with four novel metabolites further increased the chemical diversity of *Aplysilla rosea*. Besides diterpenes and lactones, 11 sterols and two fatty acids were found in sponge *Aplysilla rosea* (Chapter 3). A potential of over 20 compounds could join in this chemical family as well.

In the current study, the faction AR was separated by Sephadex LH-20 gel to sub-fractions AR1-3, and only sub-fraction AR3 was chosen for the further study. In the future, the other two fractions could be carried out for further isolation and purification. Additional, the purification step flowed by Sephadex LH-20, ODS, silica gel and HPLC. In the future, the purification steps could be optimized to isolate other minor compounds.

5.2.4 Cytotoxicity induced by compounds isolated from *Aplysilla rosea*

In these 24 compounds from *Aplysilla rosea*, 12 compounds showed different cytotoxicity against three human breast cancer cell lines, five of those 12 compounds indicated selective cytotoxic effect against breast cancer cells over non-cancer breast cells (Chapter 4). In the future, in order to determine the molecular mechanisms, the mRNA expression levels of cell cycle regulating genes at the G_0/G_1 could be determined by qRT-PCR. Also, the mechanisms of these selective compounds warrant further investigation (e.g. caspase 3/7, 8 or 9). Additionally, the selectivity only showed a margin of safety with a maximum of an approximately 5-fold difference between cancer and normal cells. Therefore, *in vivo* studies need to be carried out to determine the efficacy and side effects. A combination of in intro and in vivo study would generate potential lead compounds for the development of therapeutic agents for the breast cancer treatment.

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APPENDICES

Appendix A1: Stock solution

Phosphate Buffered Saline (PBS) (10×) 1 litre

KCl 2 g

KH_2PO_4 2 g

NaCl 80 g

Na_2HPO_4 11.5 g

Stored at room temperature

MTT Solution (5 mg/ml)

Dissolve 250 mg MTT in 50 ml 1 × PBS

Filter sterilised and aliquot to 5 ml each

Stored at -20°C

Trypan Blue

Dissolve 1.8 g NaCl in 180 ml MilliQ H_2O

Dissolve 0.4 g Trypan Blue in solution as above

Made up to 200 ml and filtered

Stored at 4°C

0.5% (w/v) Crystal Violet (200 ml)

Dissolve 1 g crystal violet in 200 ml of 50% (v/v) methanol (1:1 methanol and H_20)

Stored at room temperature

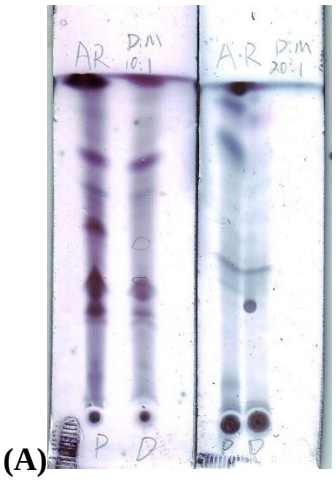
33% Acetic Acid Destain Solution (1 L)

Add 330 ml of acetic acid into 670 ml of MilliQ H_2O

Mix and stored at room temperature

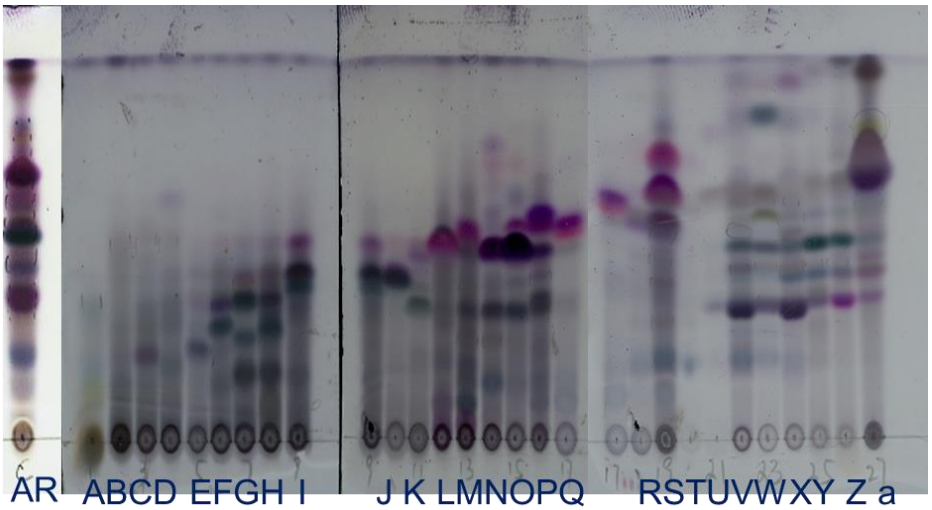
Appendix A2: TLC

The thin-layer chromatography (TLC) patterns of fraction/sub-fraction from *Aplysilla rosea* extract



(A)

(B)

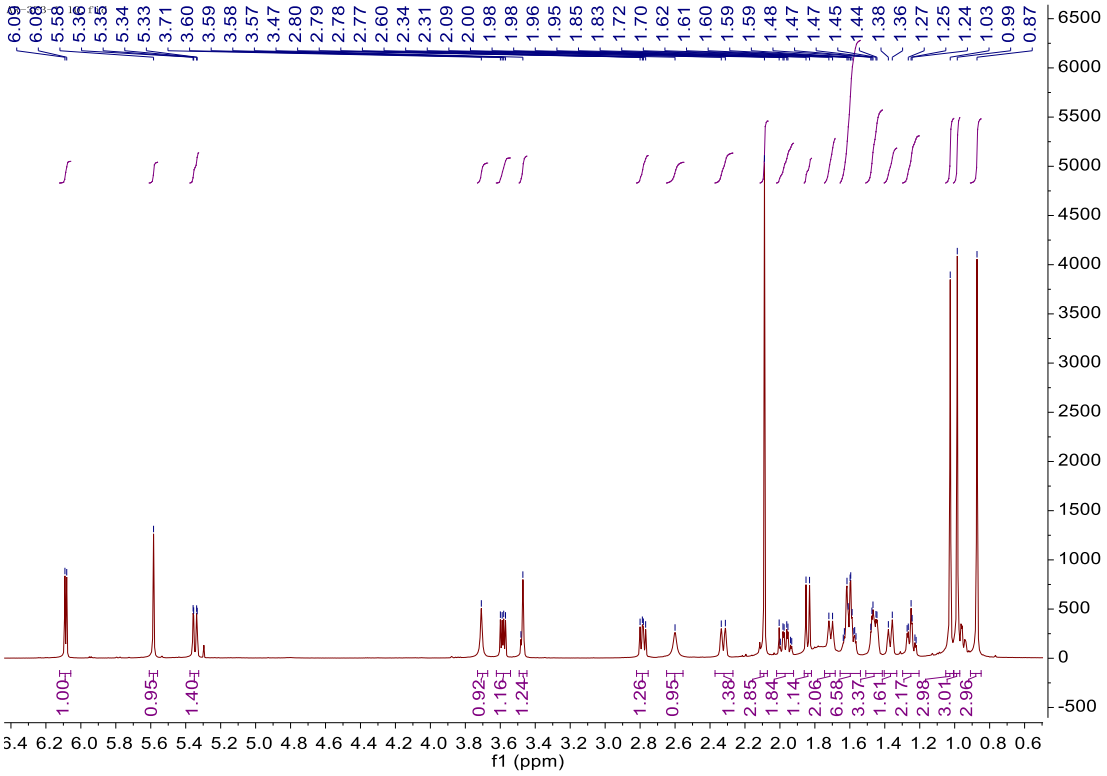


(C)

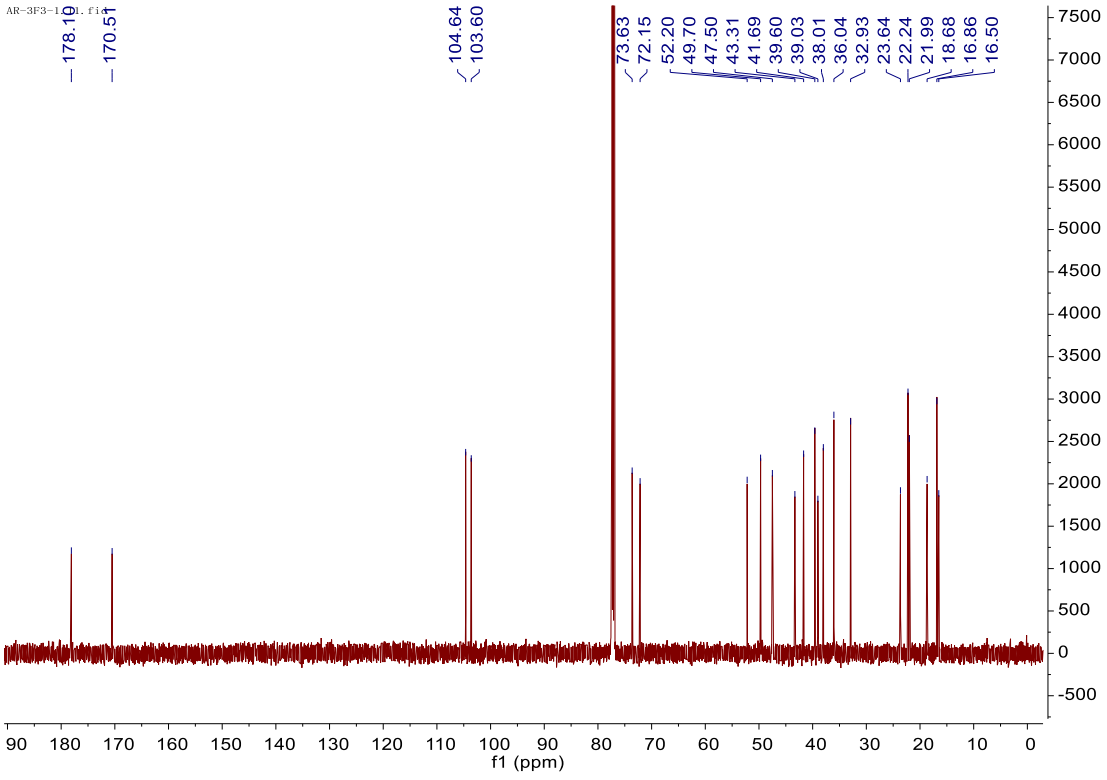
Note: (A) compare fraction 1 and 2 with two solvent systems left (DCM-MeOH, 10:1), right (DCM-MEOH, 20:1); (B) sub-fraction A, B and C in Hexane-Acetone (5:2); (C) fraction AR (pre-ODS) and fractions AR-3A-3Z (post-ODS) in Hexane-Acetone (5:2).

Appendix AR: NMR spectrum

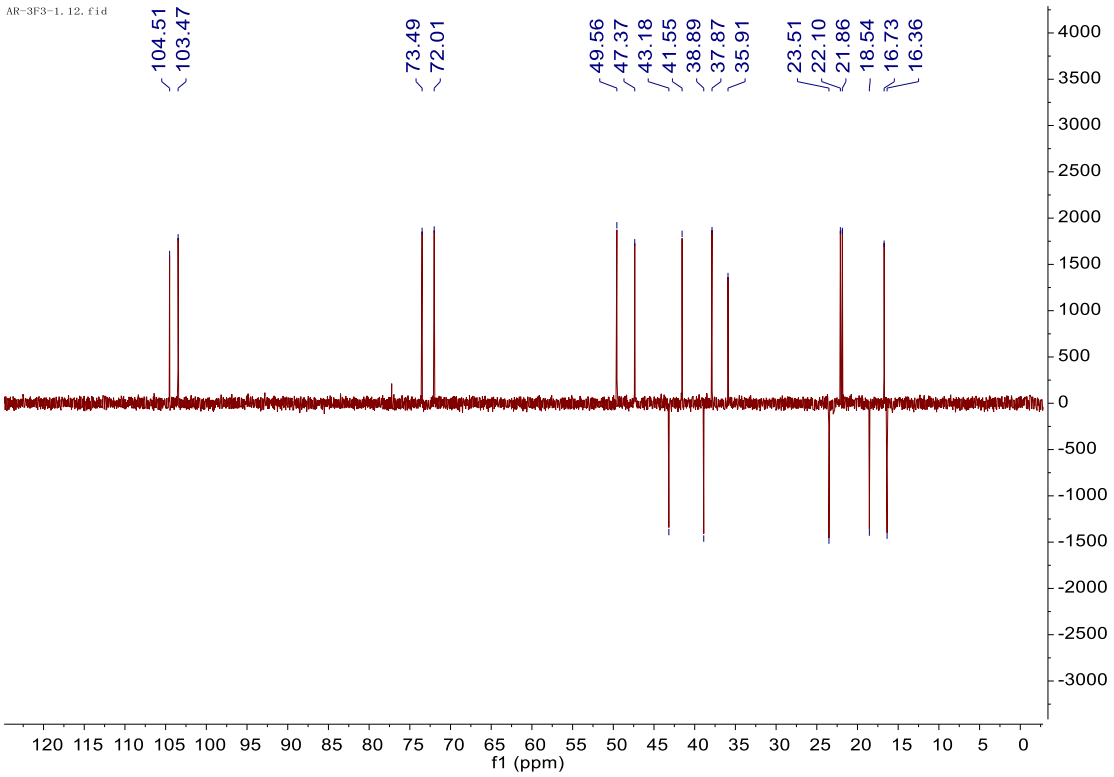
AR11 ^{1}H NMR spectrum of compound 1 in $CDCl_3$



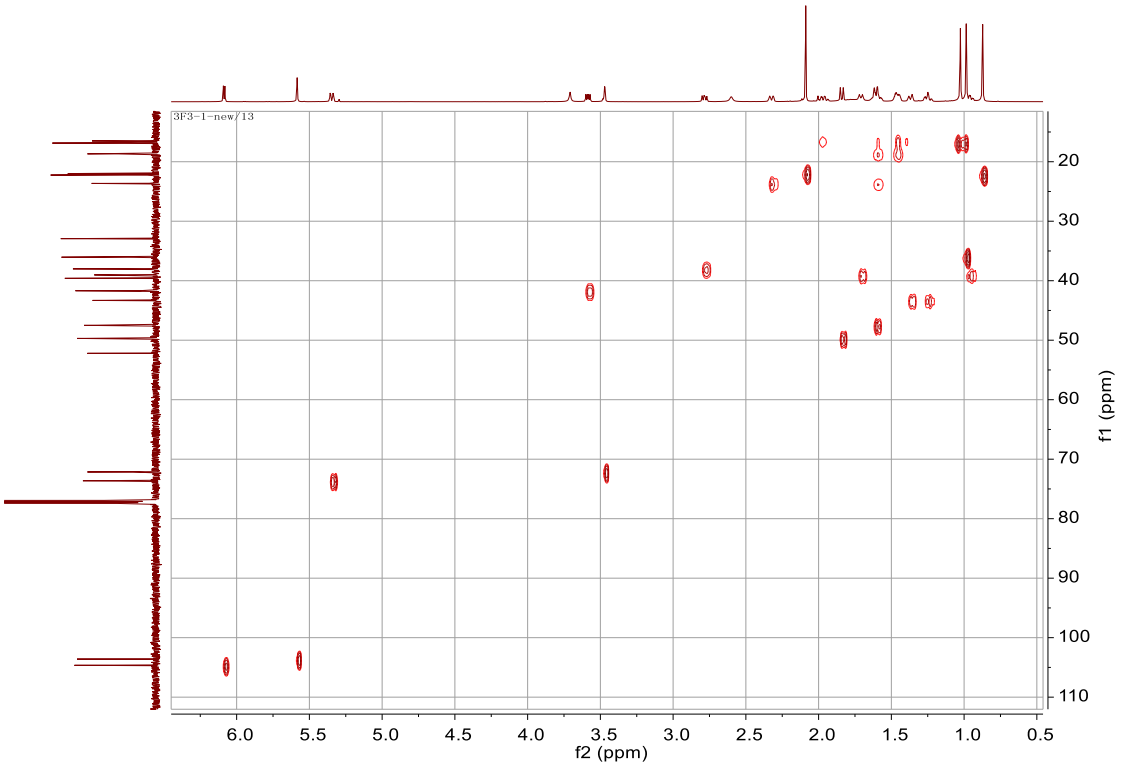
AR12 ^{13}C NMR spectrum of compound 1 in $CDCl_3$



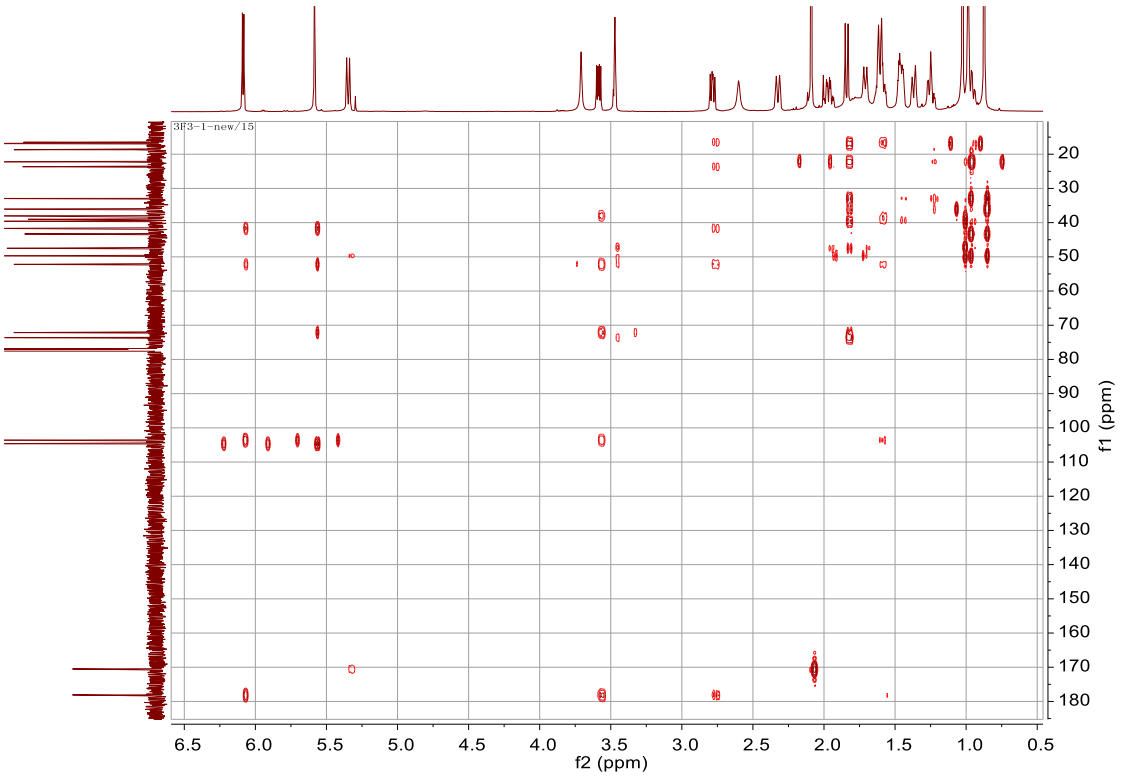
AR13 DEPT spectrum of compound 1 in $CDCl_3$



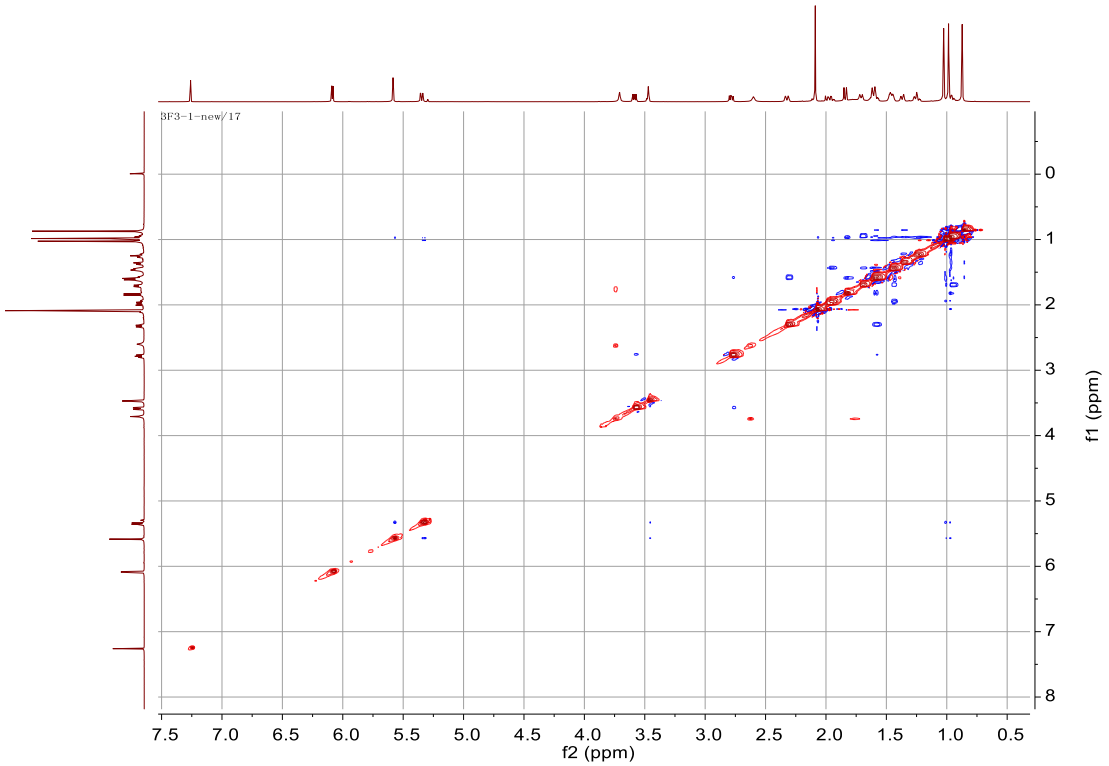
AR14 HSQC spectrum of compound 1 in $CDCl_3$



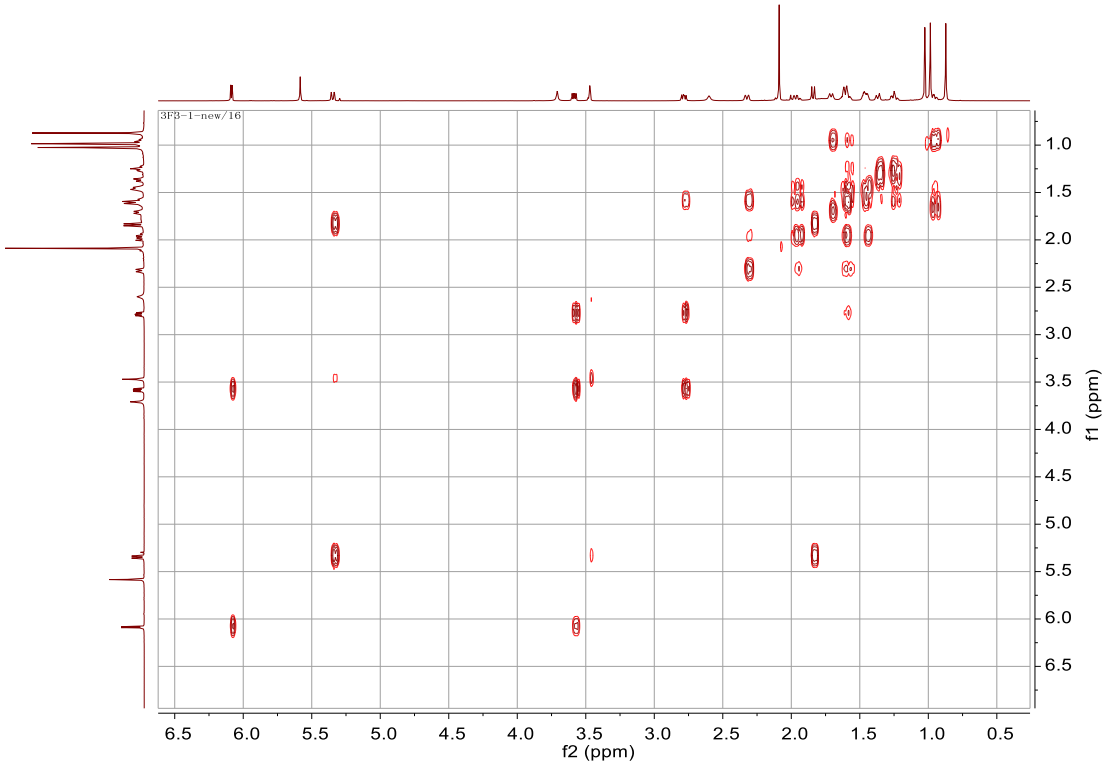
AR15 HMBC spectrum of compound 1 in $CDCl_3$



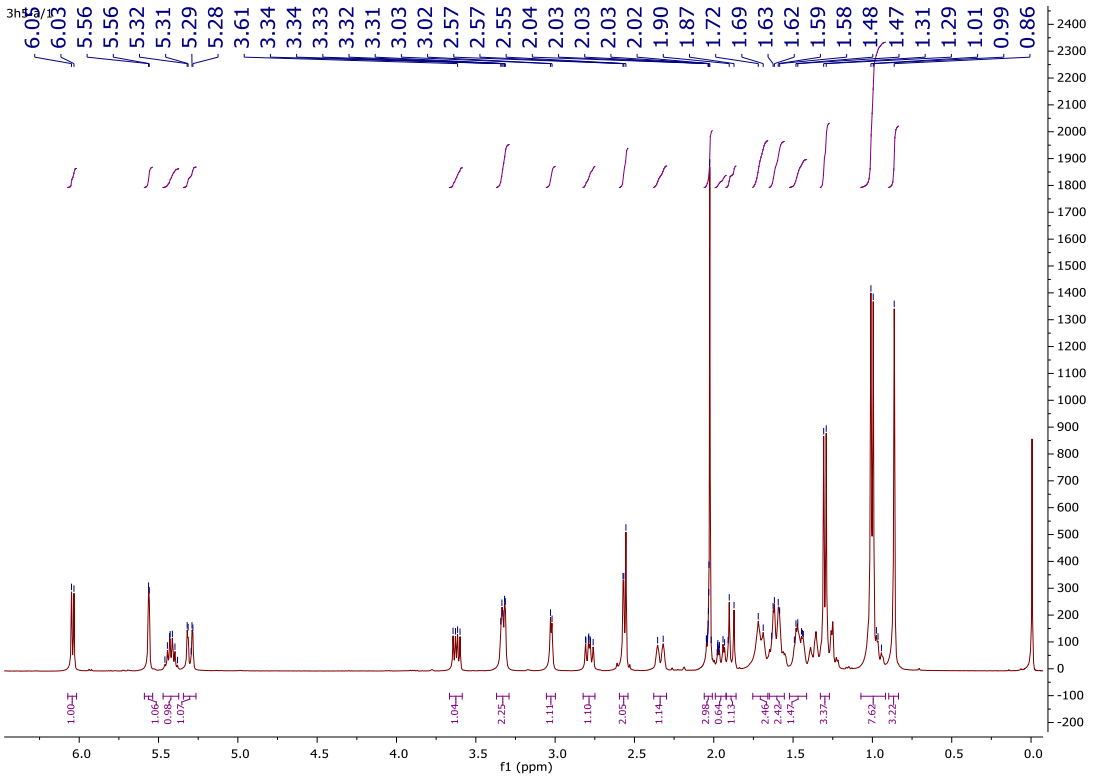
AR16 NOESY spectrum of compound 1 in $CDCl_3$



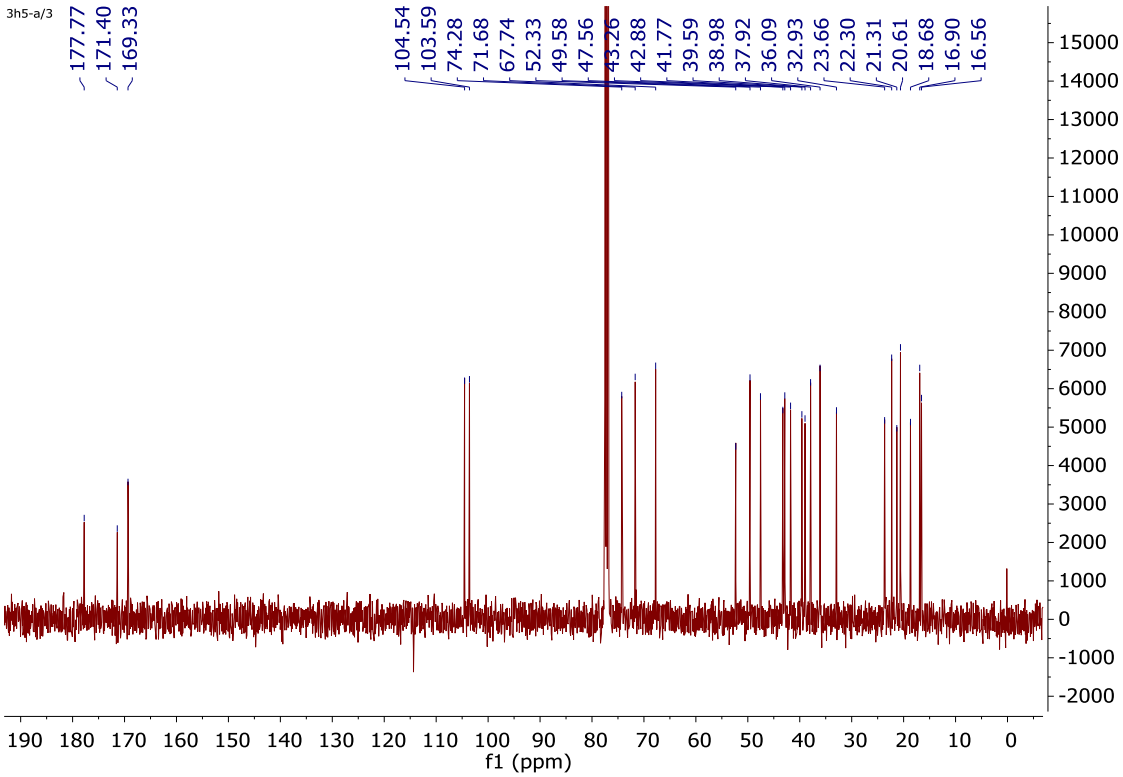
AR17 COSY spectrum of compound 1 in $CDCl_3$



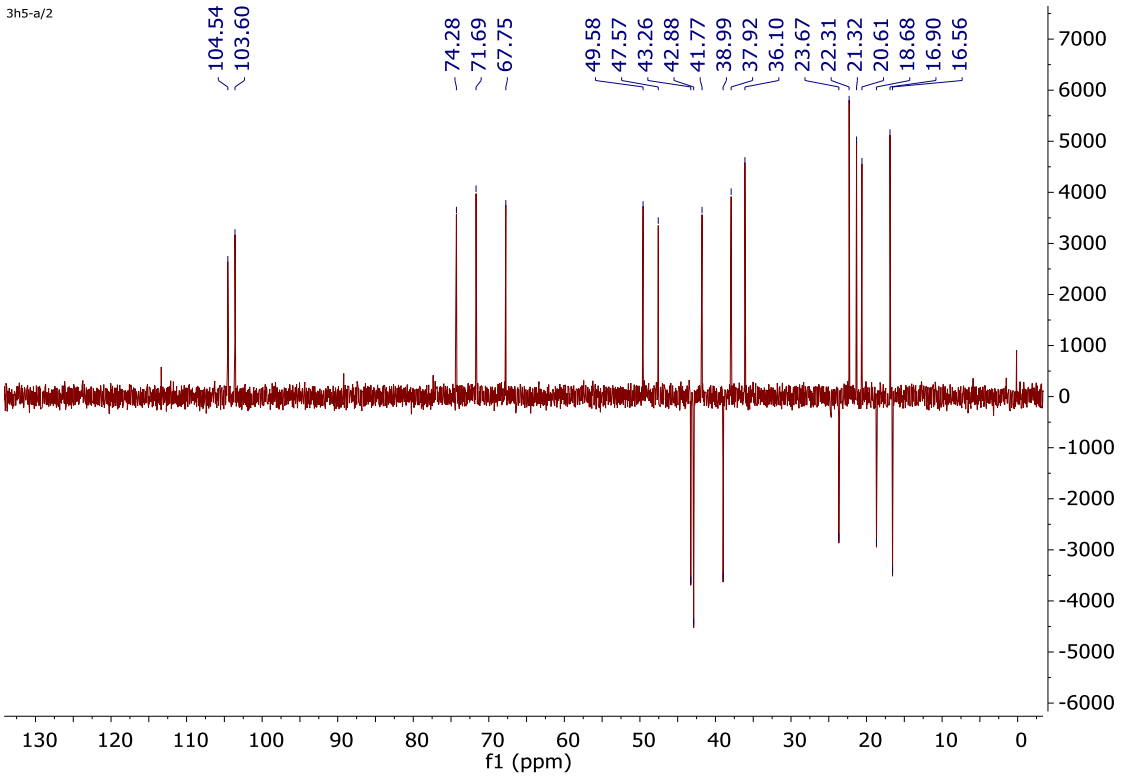
AR21 ^{1}H NMR spectrum of compound 2 in $CDCl_3$



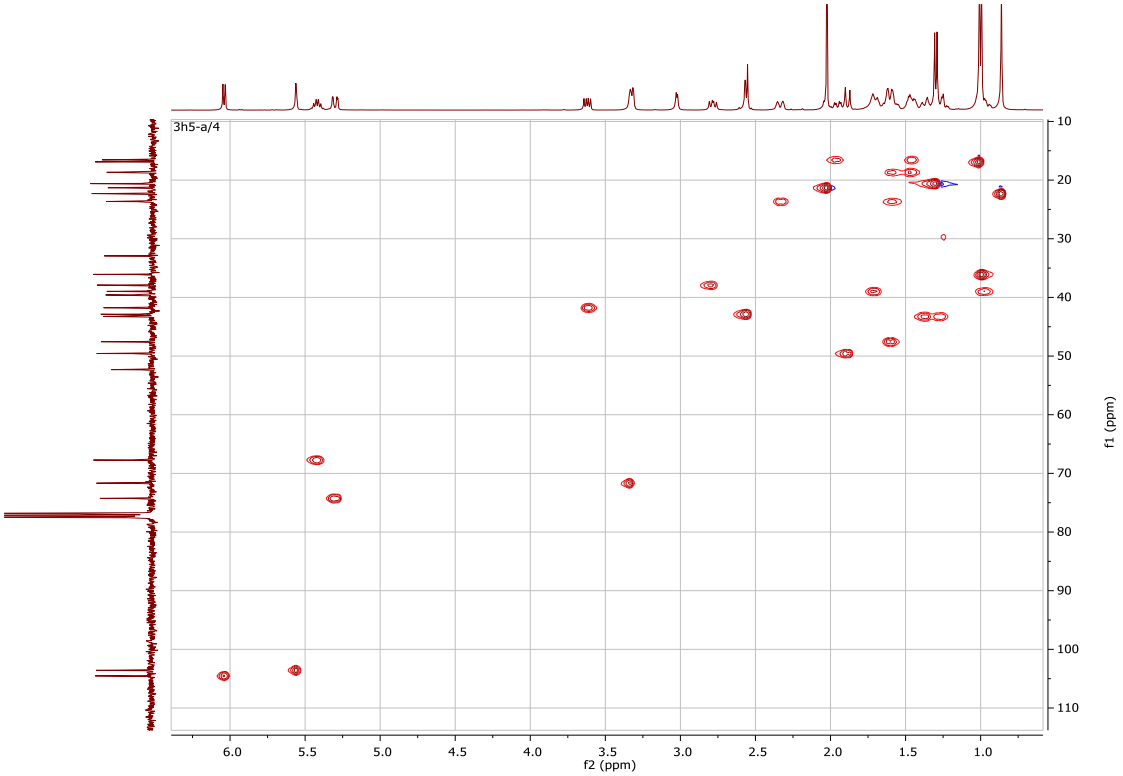
AR22 ^{13}C NMR spectrum of compound 2 in $CDCl_3$



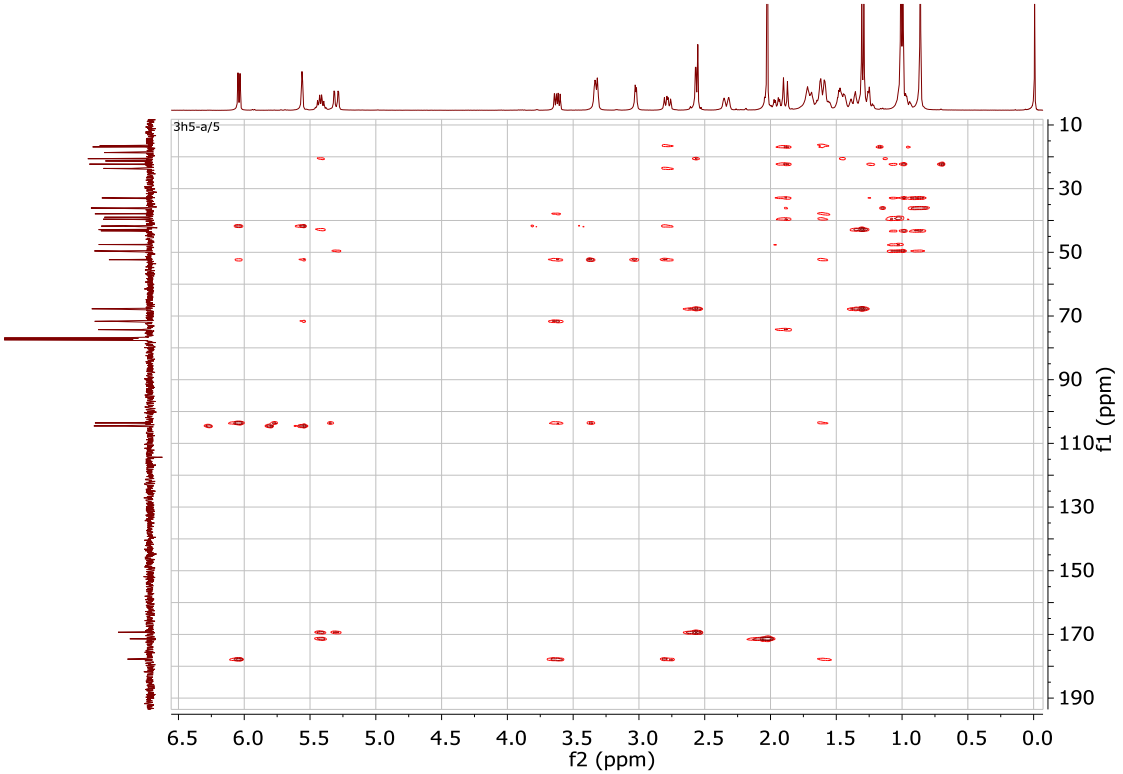
AR23 DEPT spectrum of compound 2 in $CDCl_3$



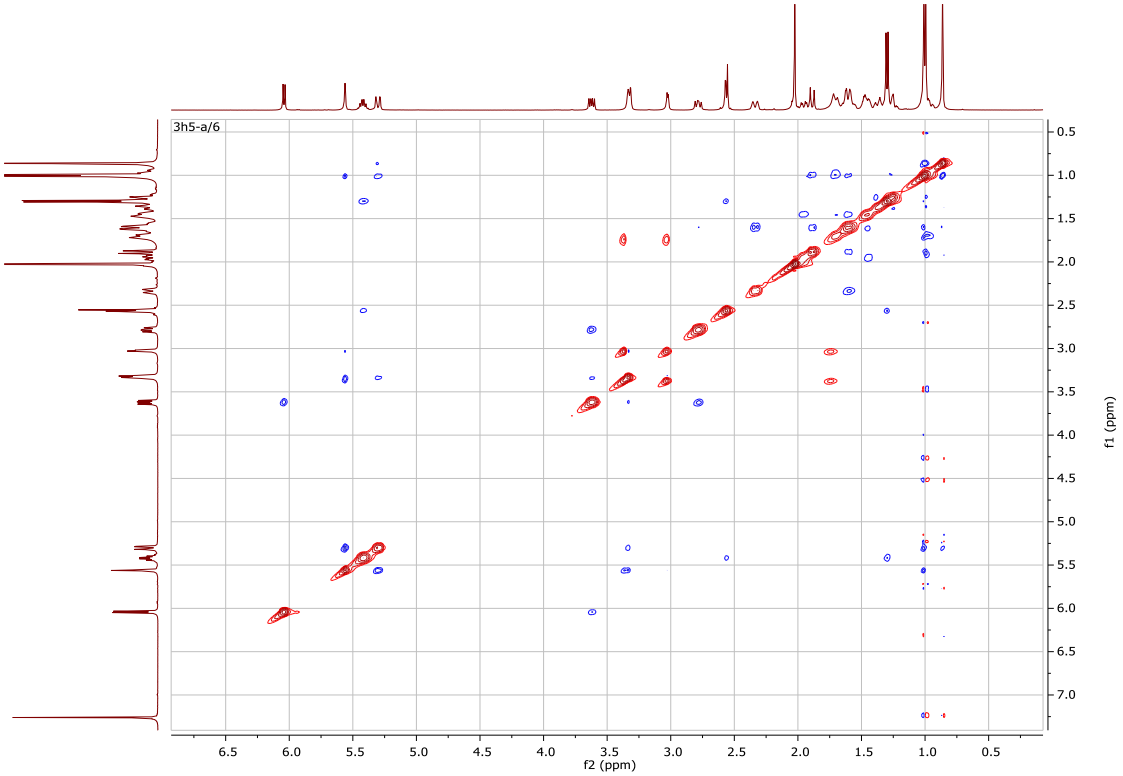
AR24 HSQC spectrum of compound 2 in $CDCl_3$



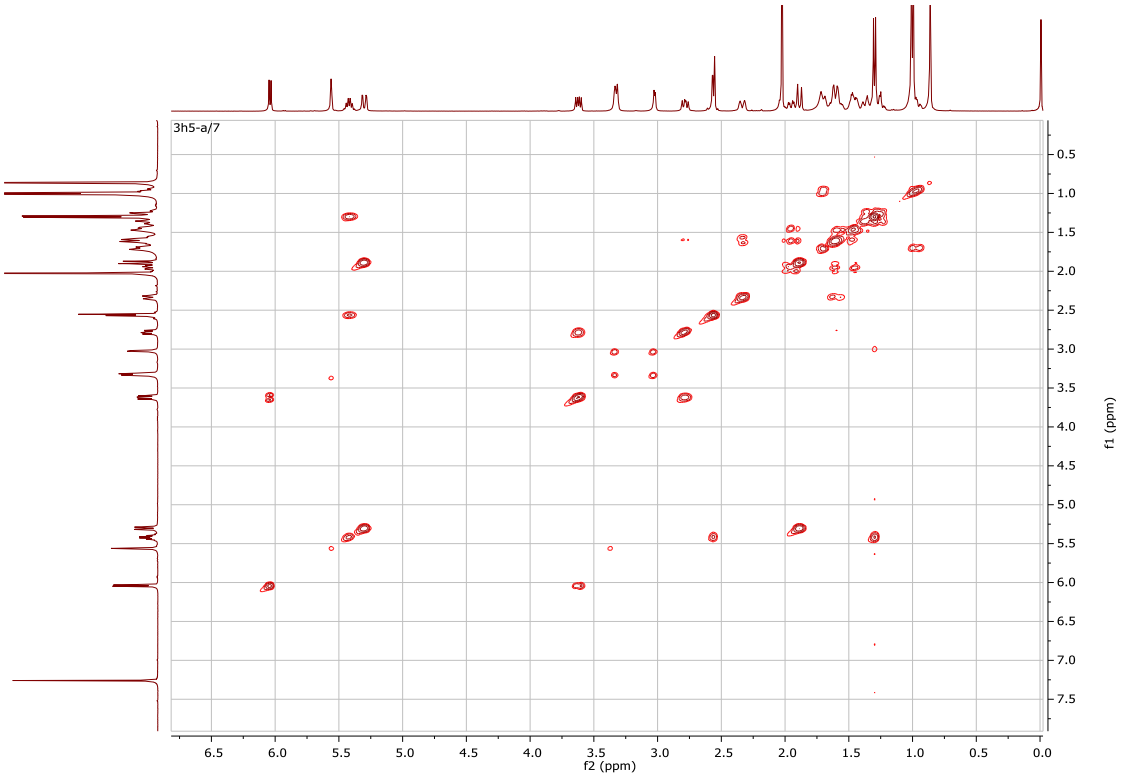
AR25 HMBC spectrum of compound 2 in $CDCl_3$



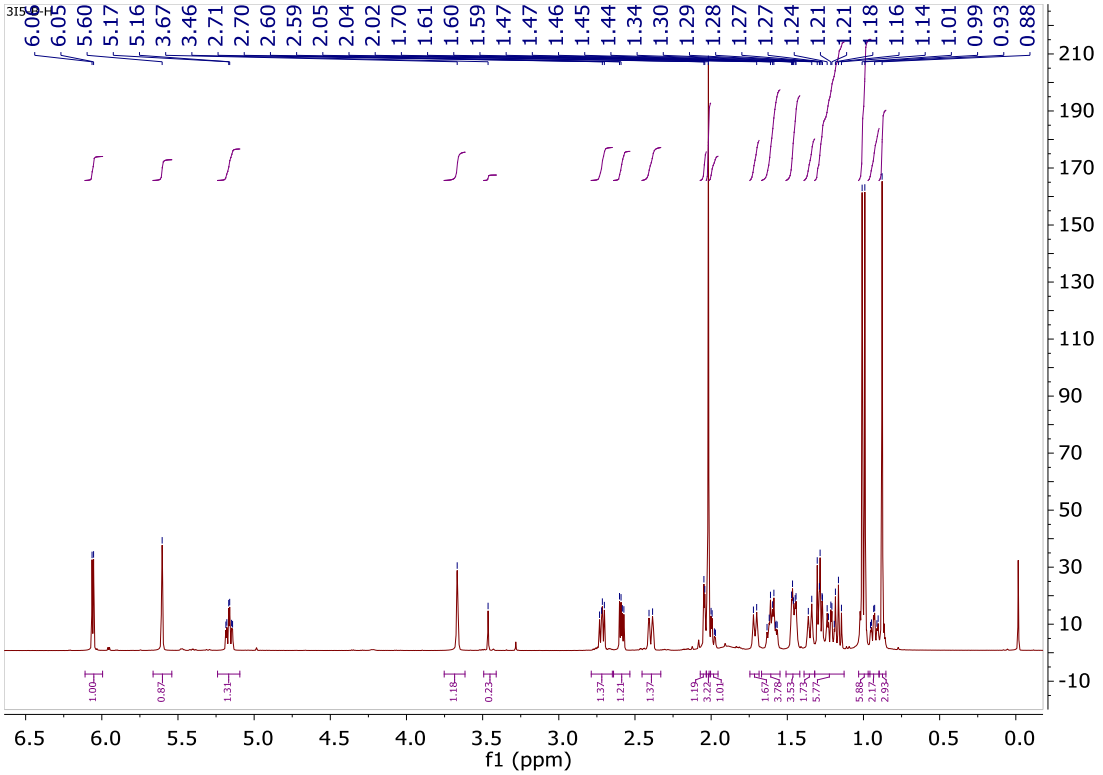
AR26 NOESY spectrum of compound 2 in $CDCl_3$



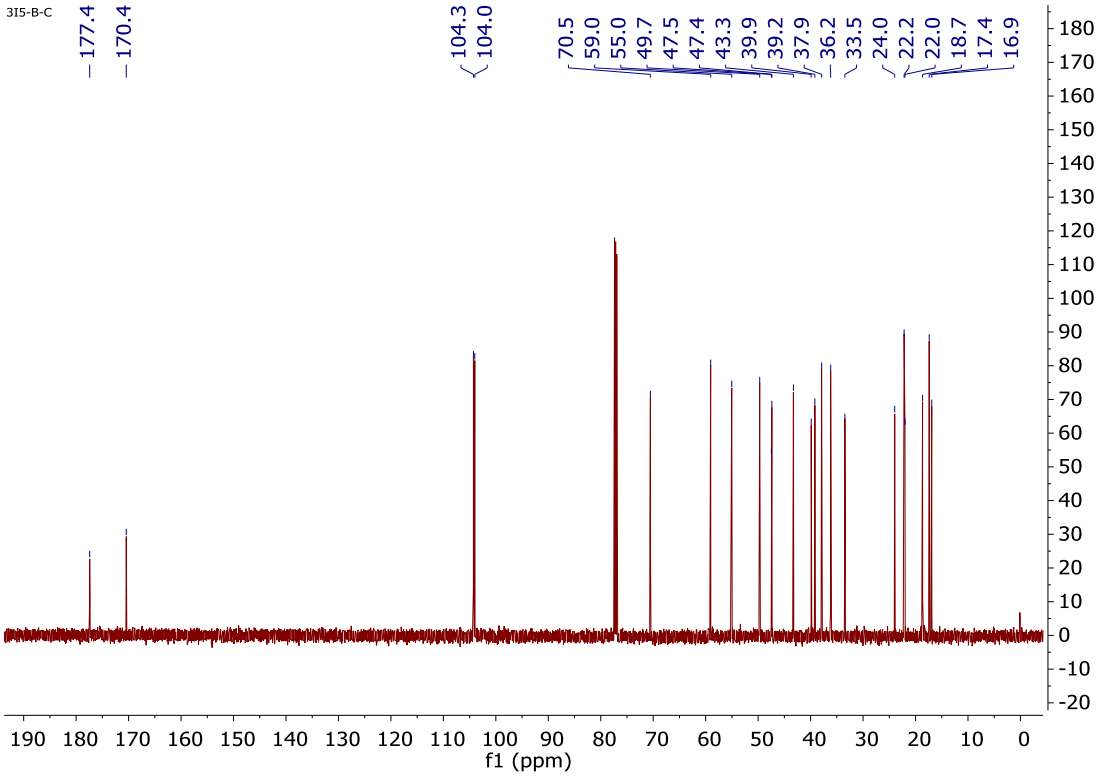
AR27 COSY spectrum of compound 2 in $CDCl_3$



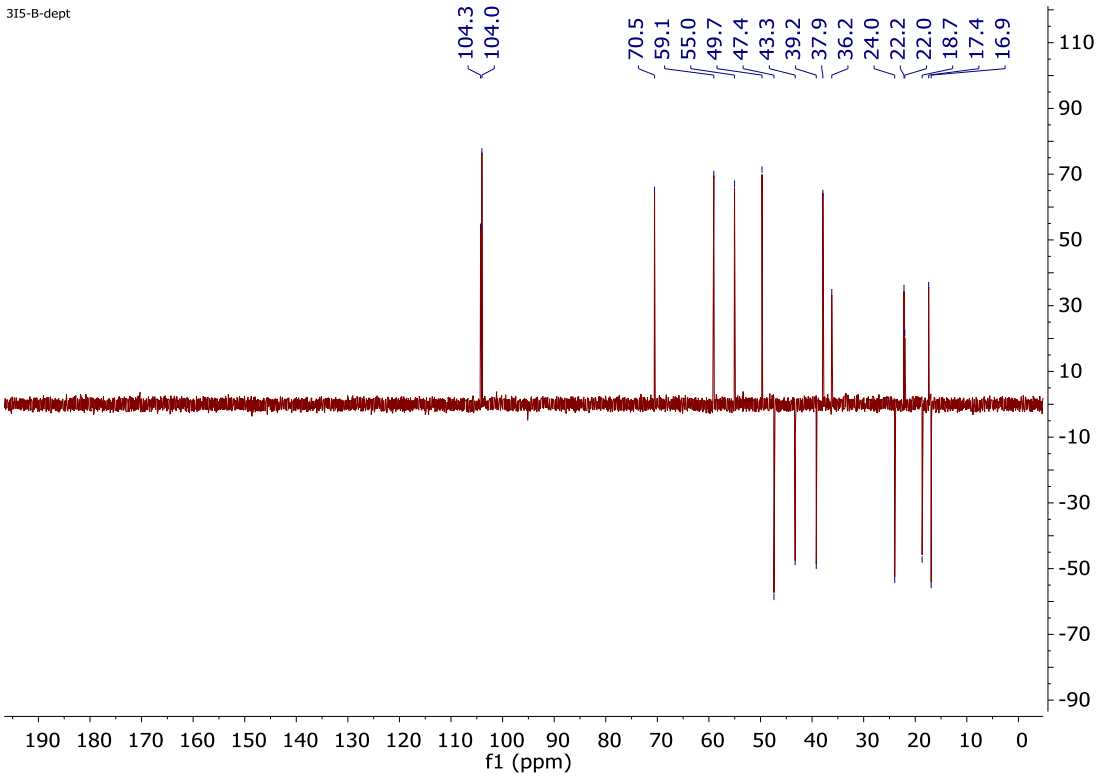
AR31 ^{1}H NMR spectrum of compound 3 in $CDCl_3$



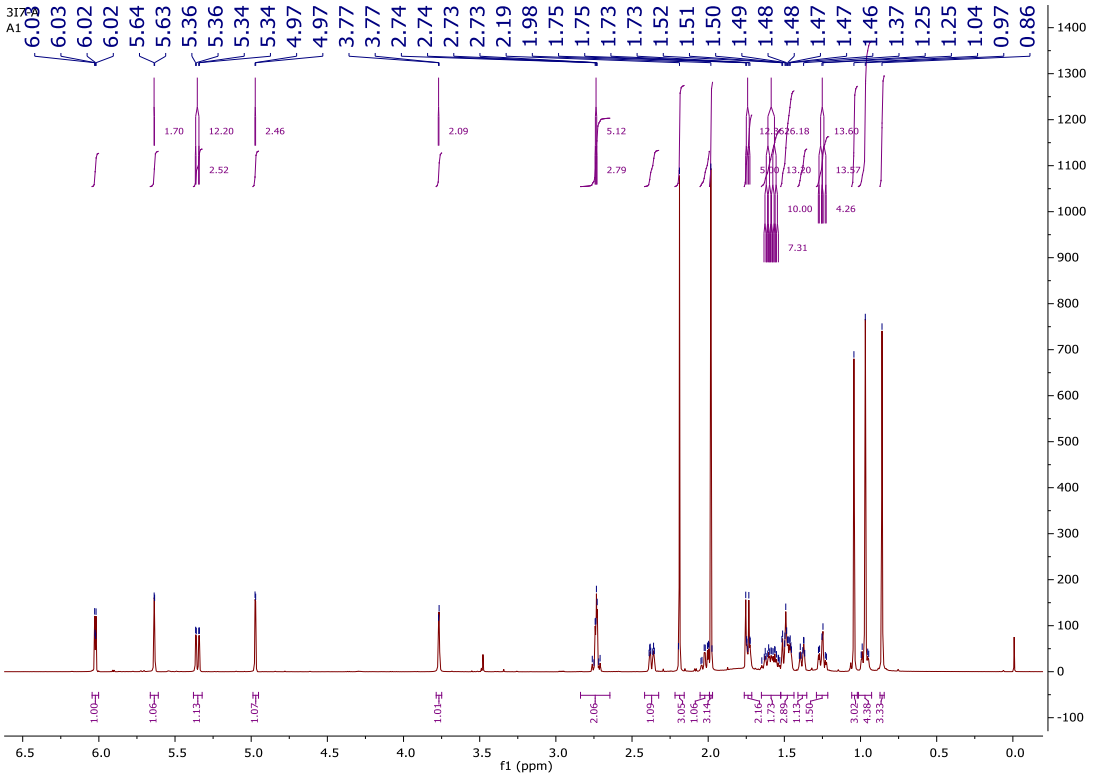
AR32 ^{13}C NMR spectrum of compound 3 in $CDCl_3$



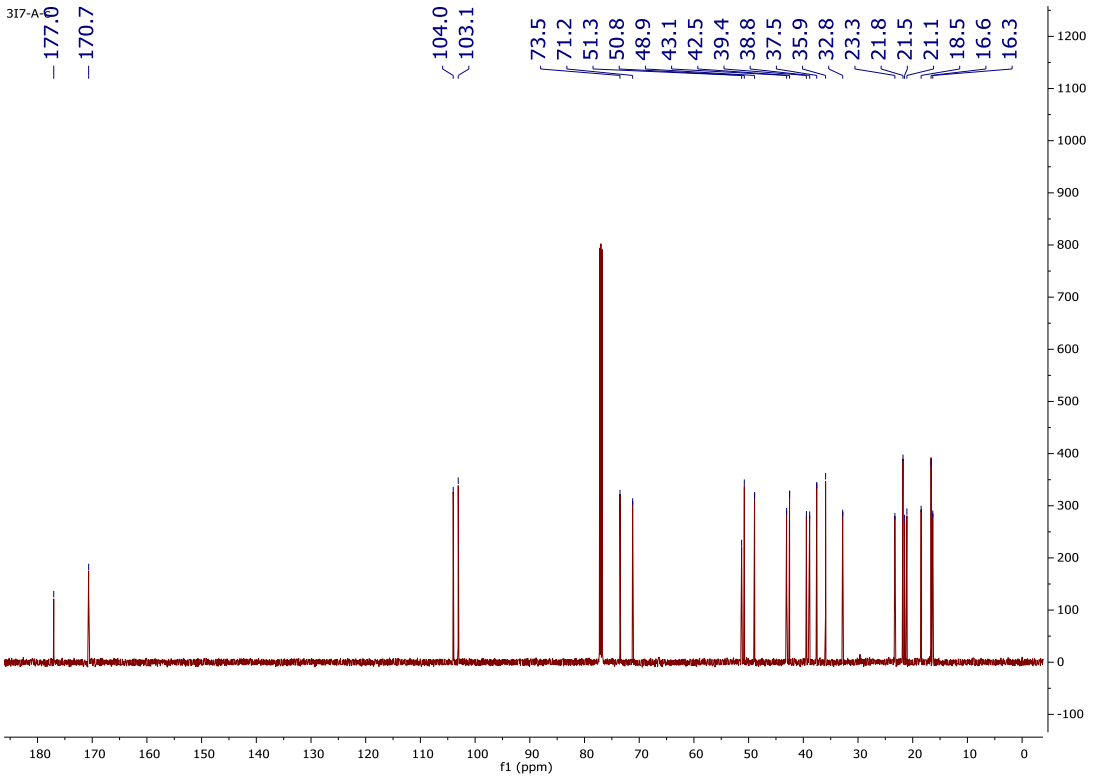
AR33 DEPT spectrum of compound 3 in $CDCl_3$



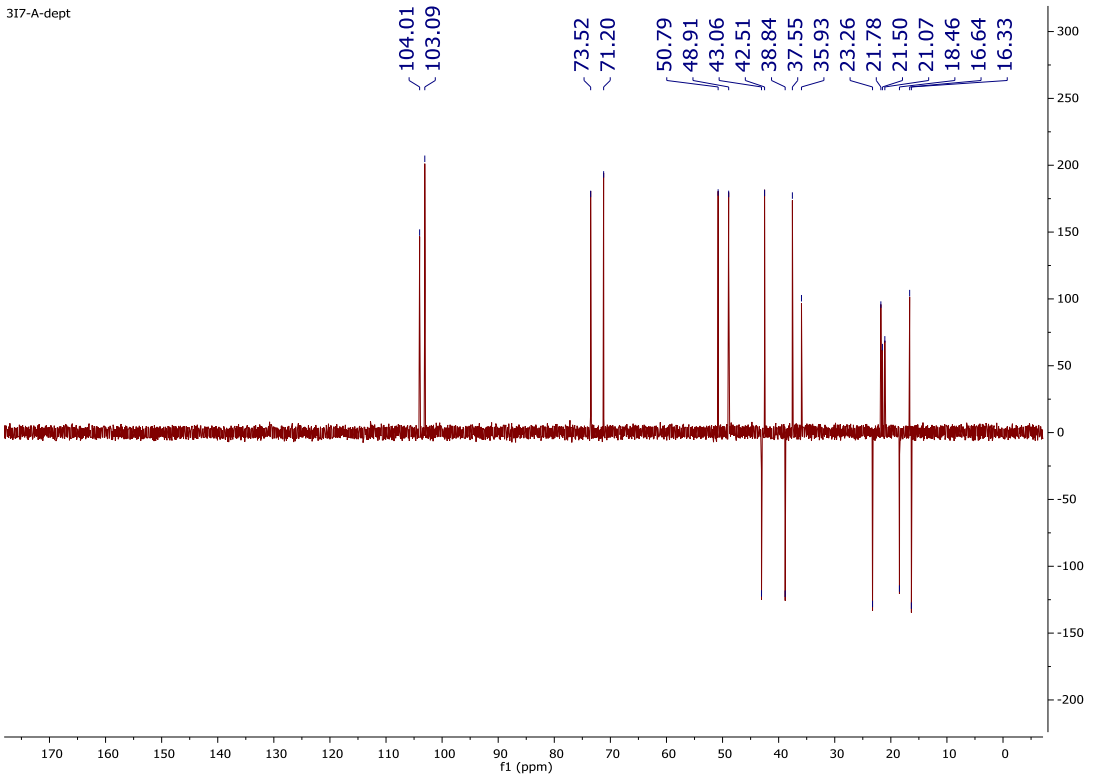
AR41 ^{1}H NMR spectrum of compound 4 in $CDCl_3$



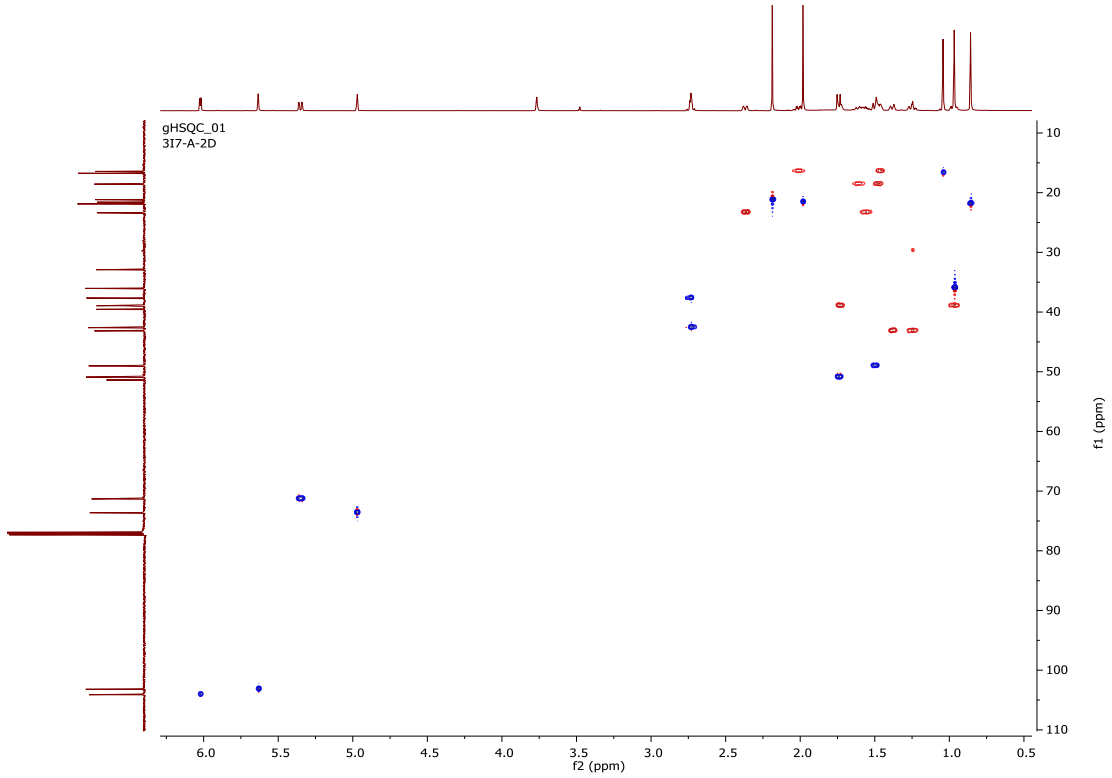
AR42 ^{13}C NMR spectrum of compound 4 in $CDCl_3$



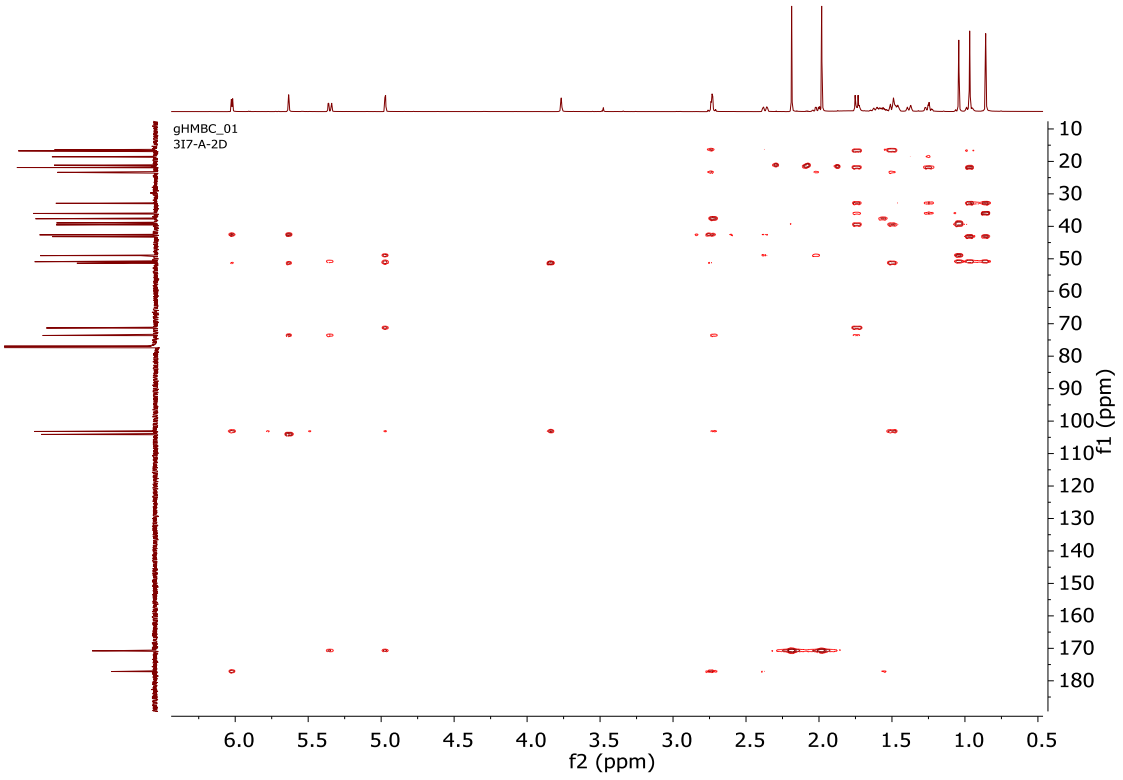
AR43 DEPT spectrum of compound 4 in $CDCl_3$



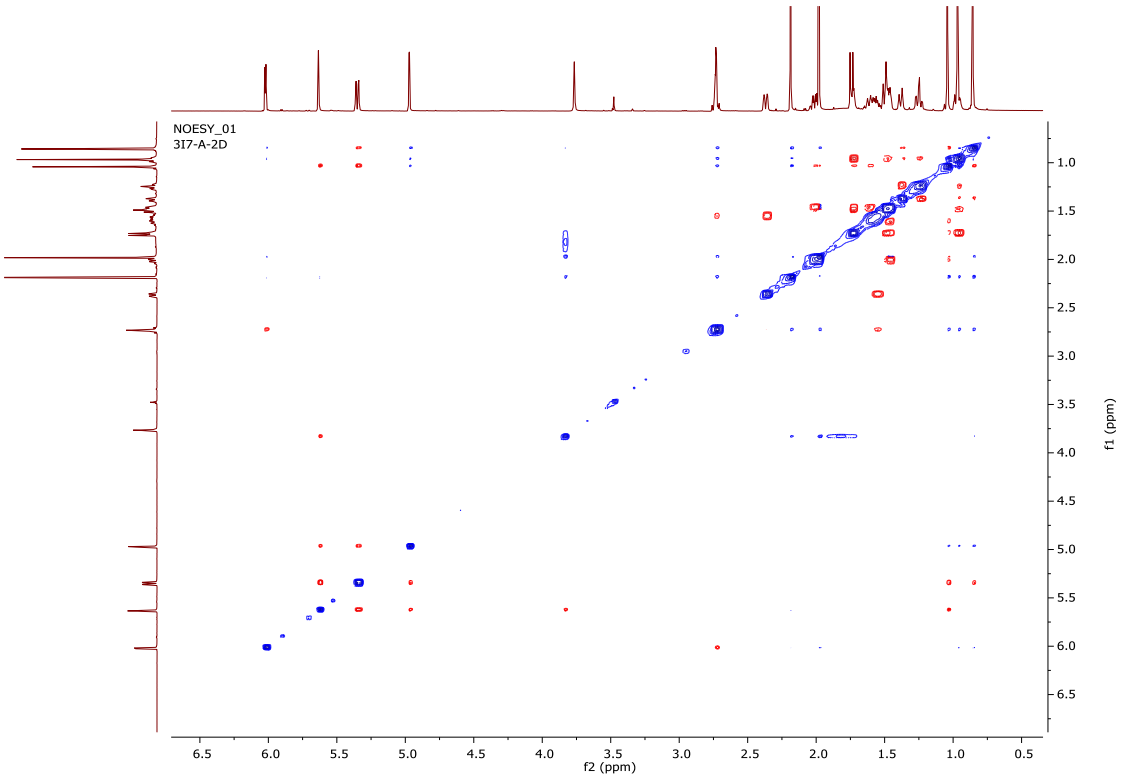
AR44 HSQC spectrum of compound 4 in $CDCl_3$



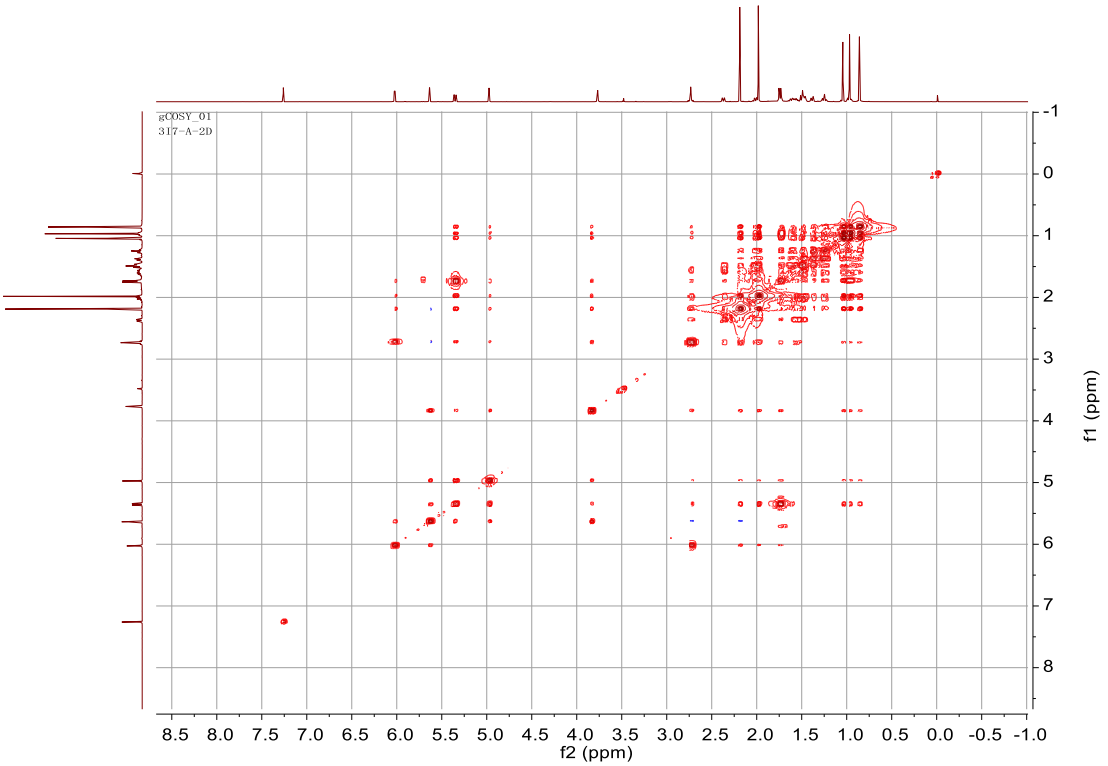
AR45 HMBC spectrum of compound 4 in $CDCl_3$



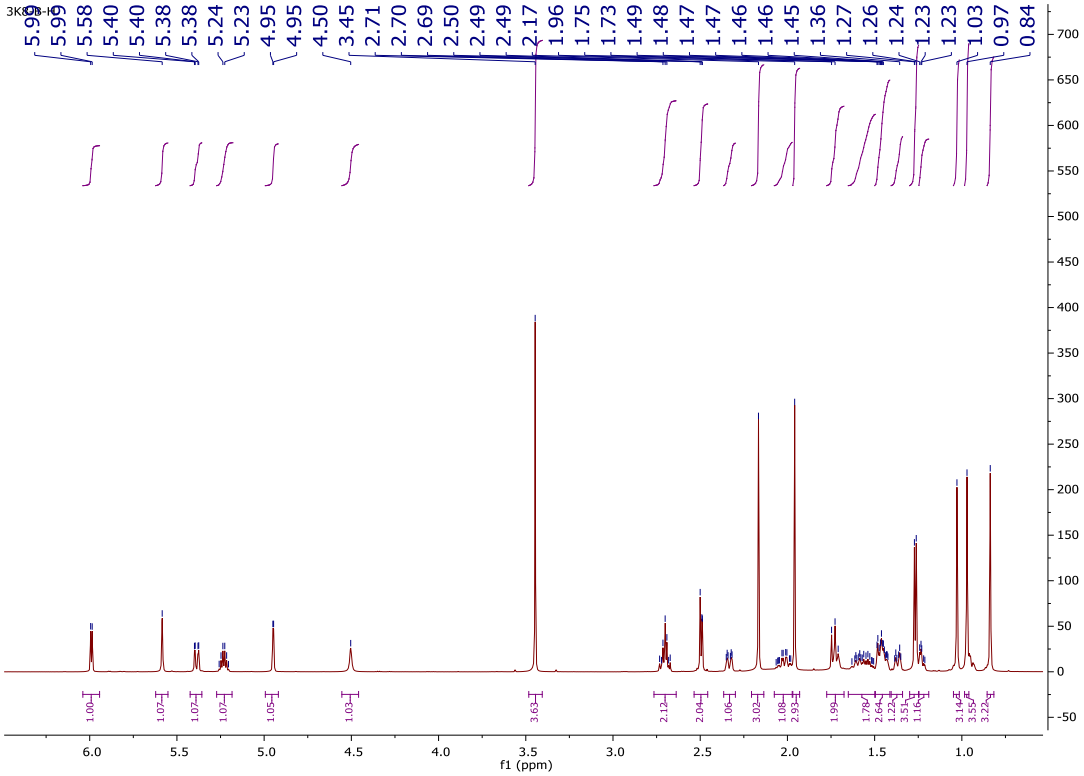
AR46 NOESY spectrum of compound 4 in $CDCl_3$



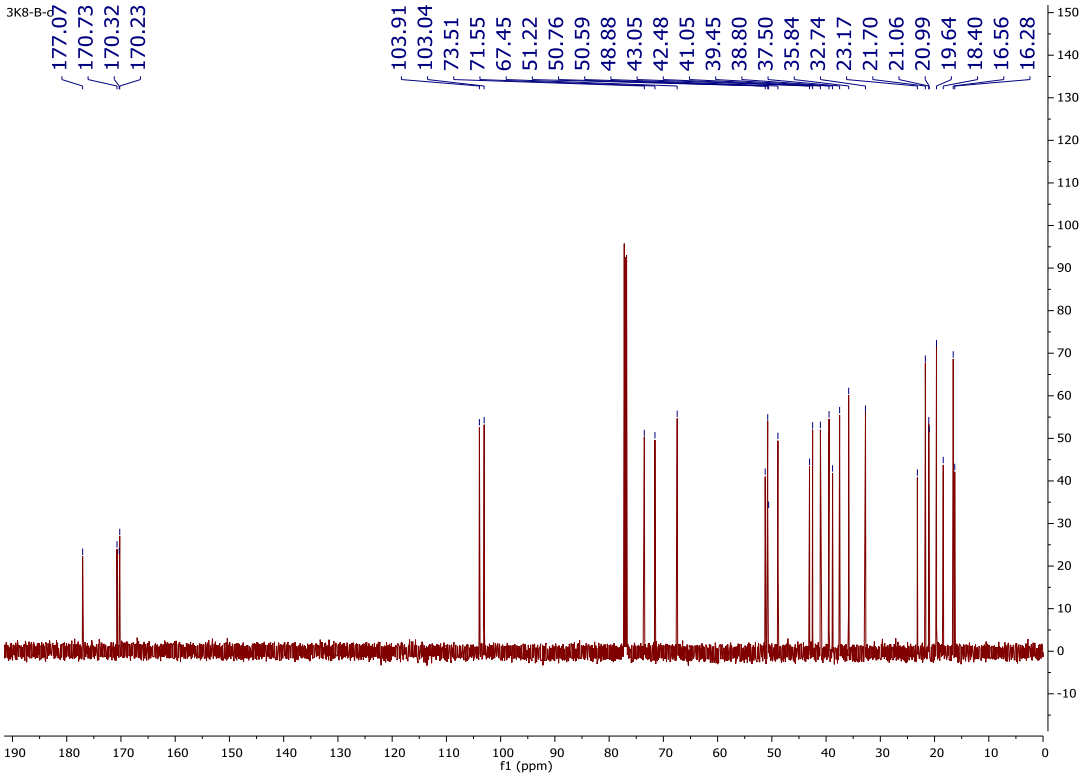
AR47 COSY spectrum of compound 4 in $CDCl_3$



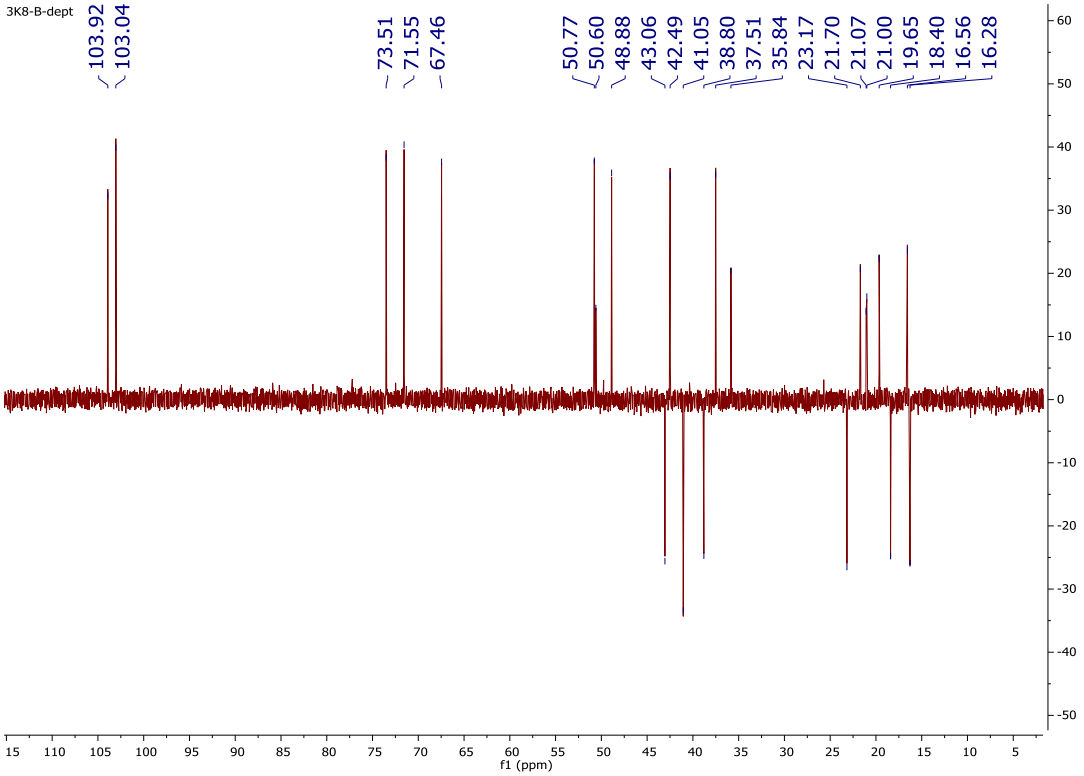
AR51 ^{1}H NMR spectrum of compound 5 in $CDCl_3$



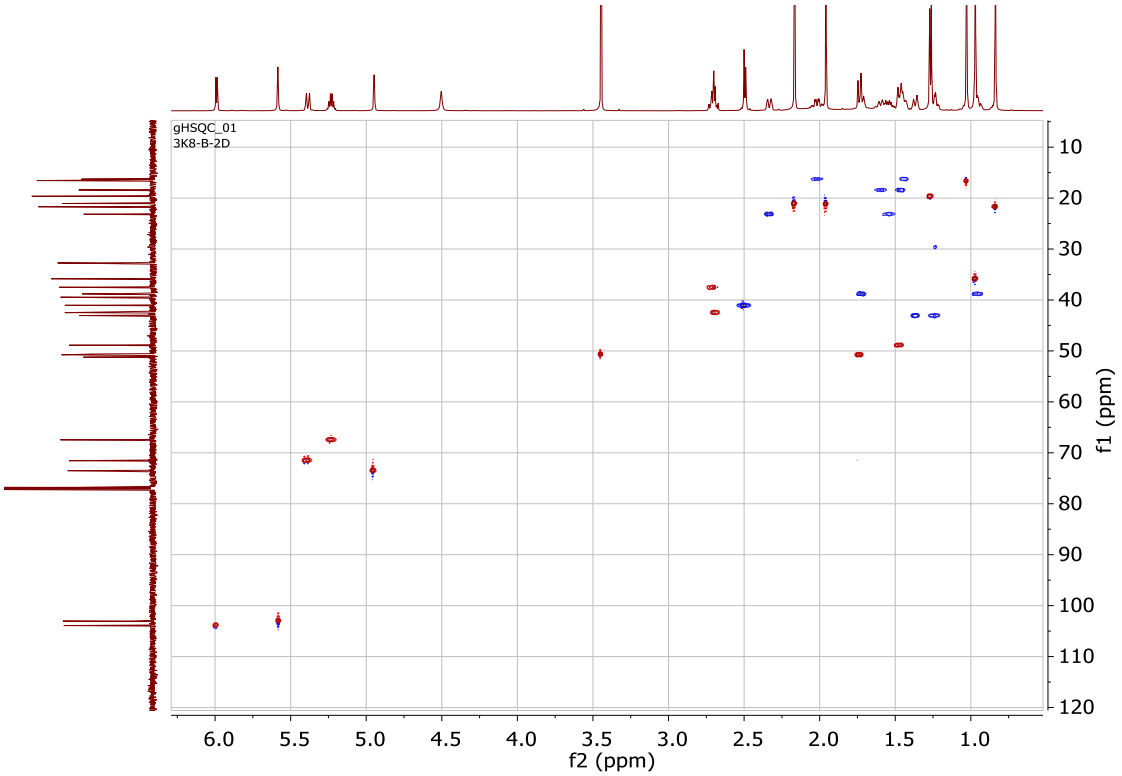
AR52 ^{13}C NMR spectrum of compound 5 in $CDCl_3$



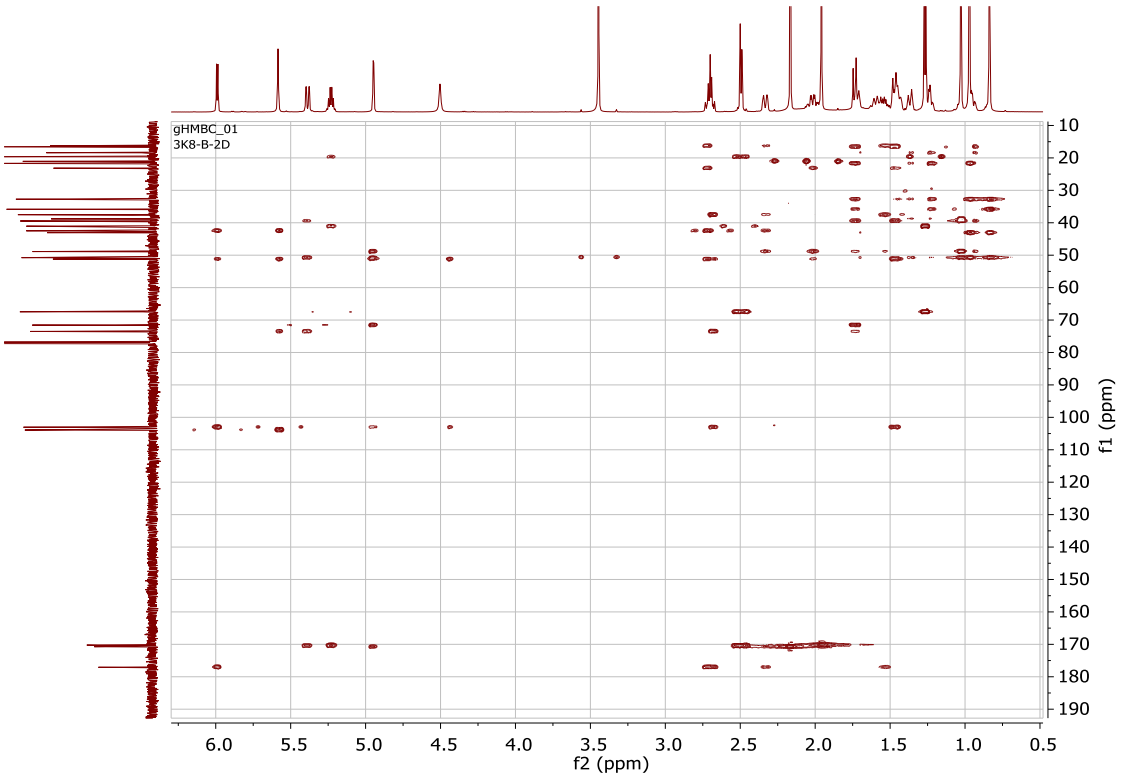
AR53 DEPT spectrum of compound 5 in $CDCl_3$



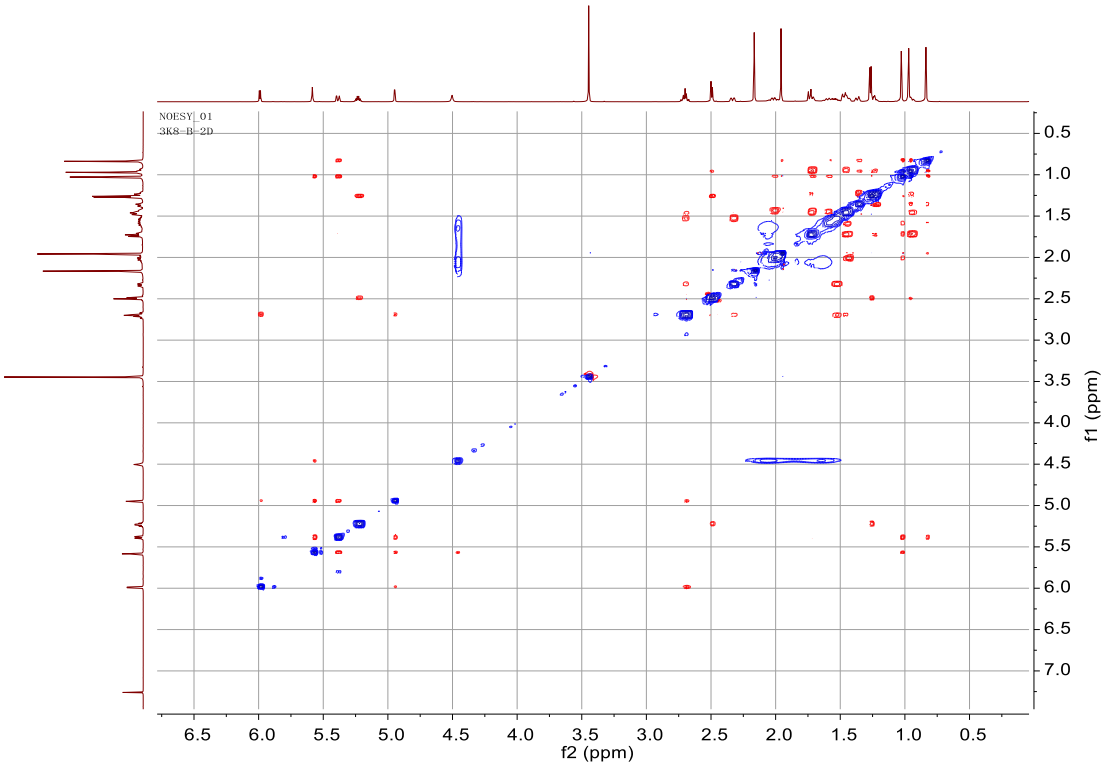
AR54 HSQC spectrum of compound 5 in $CDCl_3$



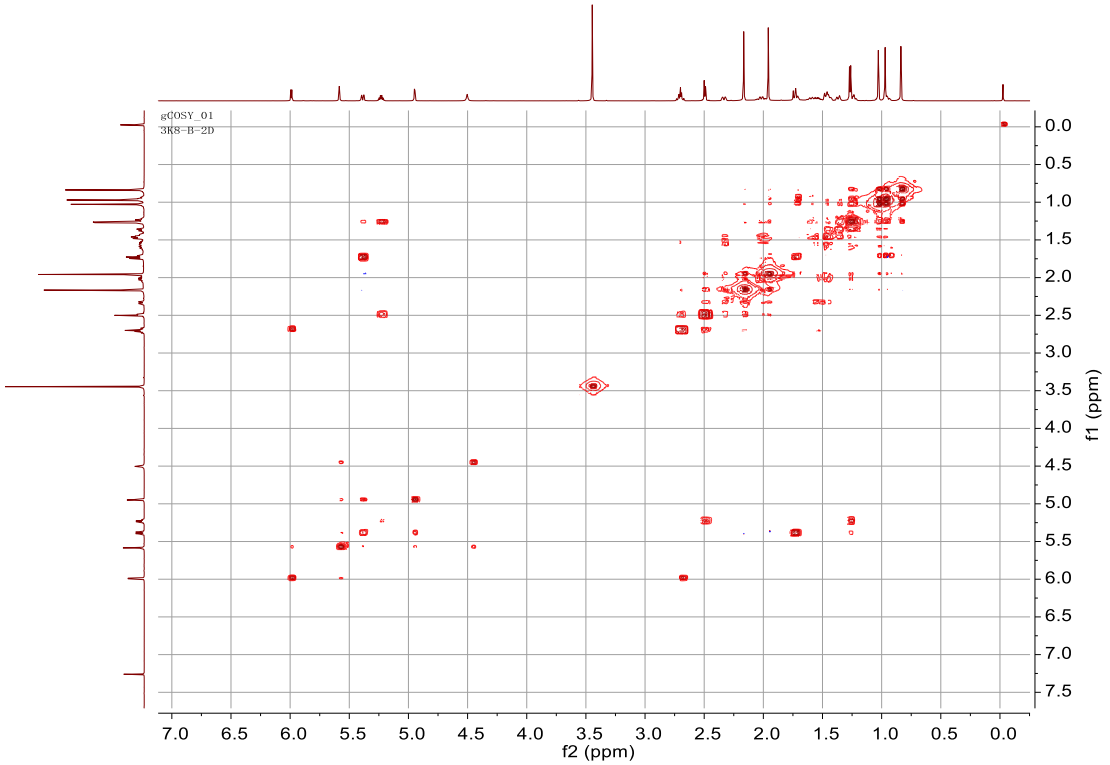
AR55 HMBC spectrum of compound 5 in $CDCl_3$



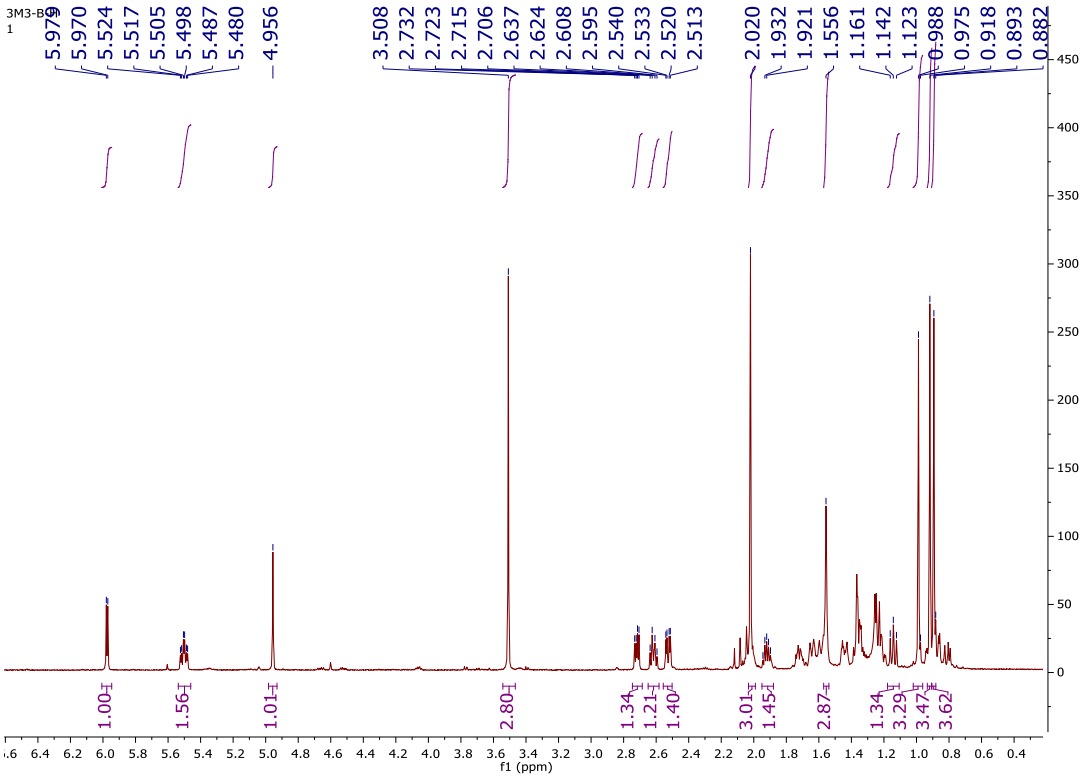
AR56 NOESY spectrum of compound 5 in $CDCl_3$



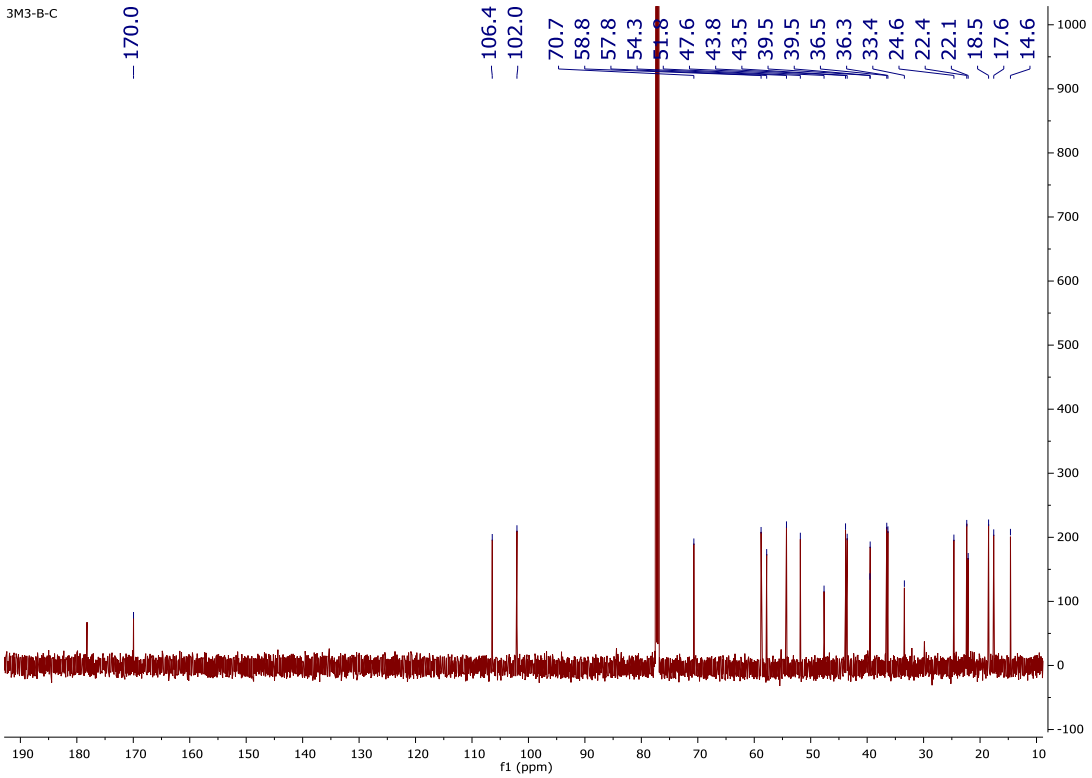
AR57 COSY spectrum of compound 5 in $CDCl_3$



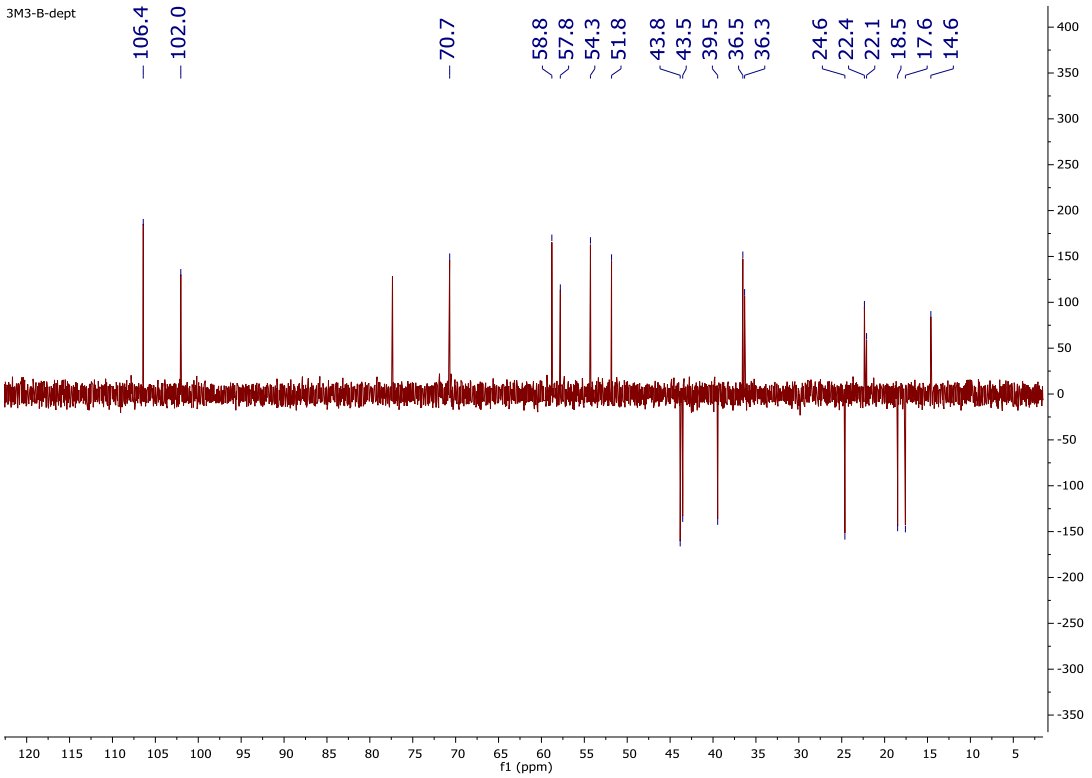
AR61 ^{1}H NMR spectrum of compound 6 in $CDCl_3$



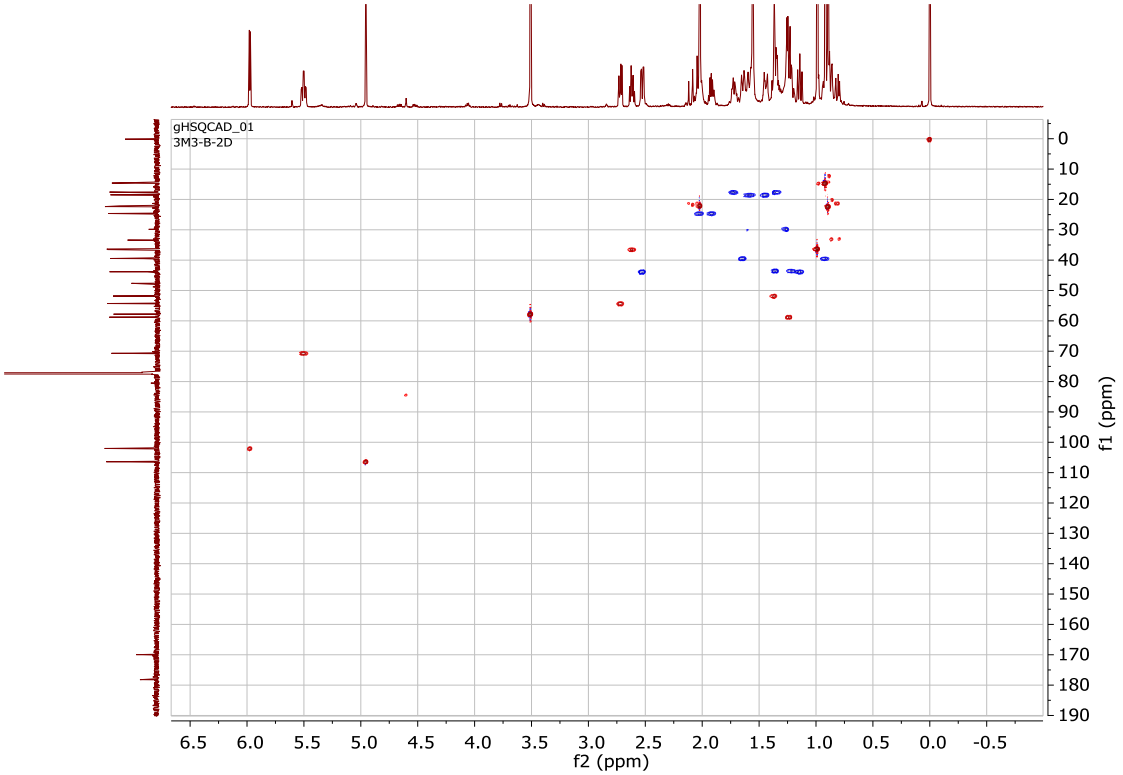
AR62 ^{13}C NMR spectrum of compound 6 in $CDCl_3$



AR63 DEPT spectrum of compound 6 in $CDCl_3$

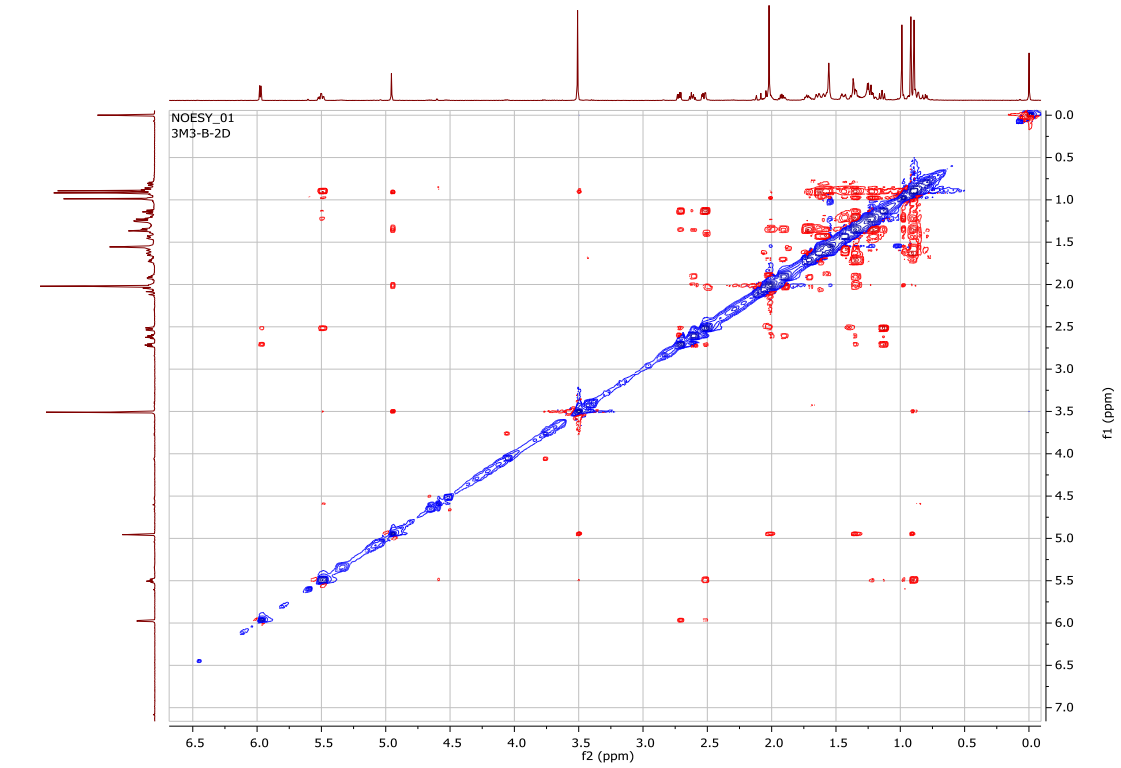


AR64 HSQC spectrum of compound 6 in $CDCl_3$

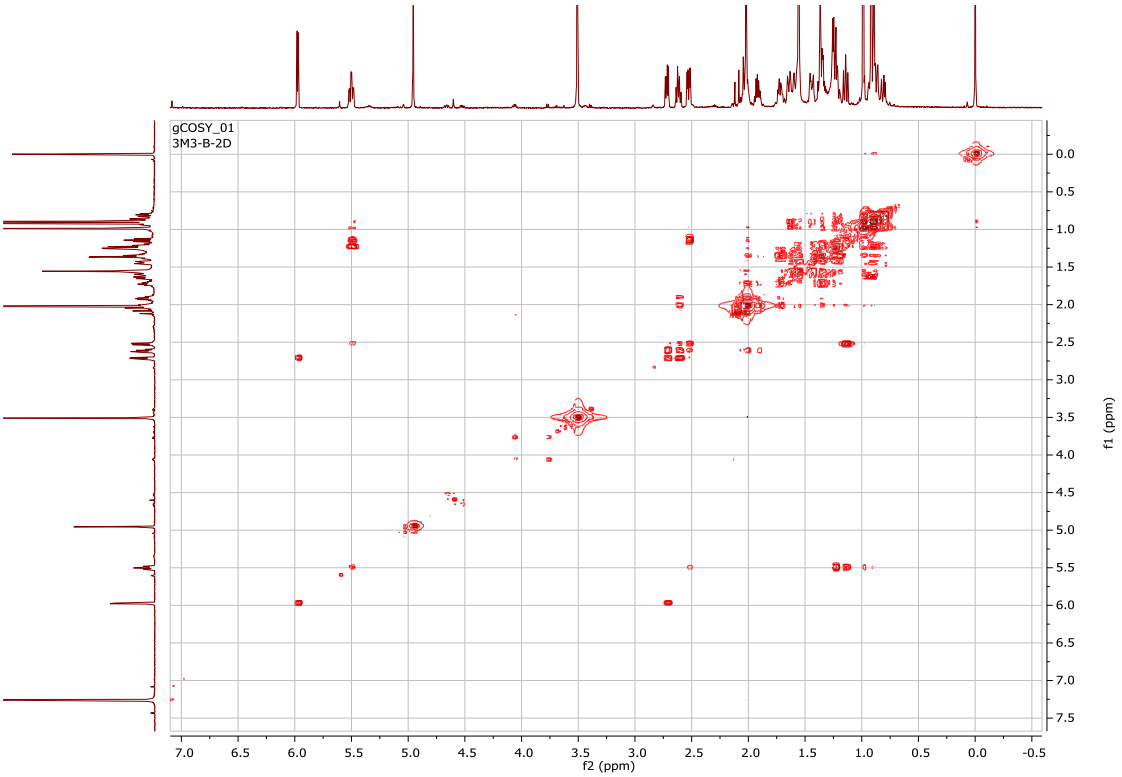


AR65 HMBC spectrum of compound 6 in $CDCl_3$

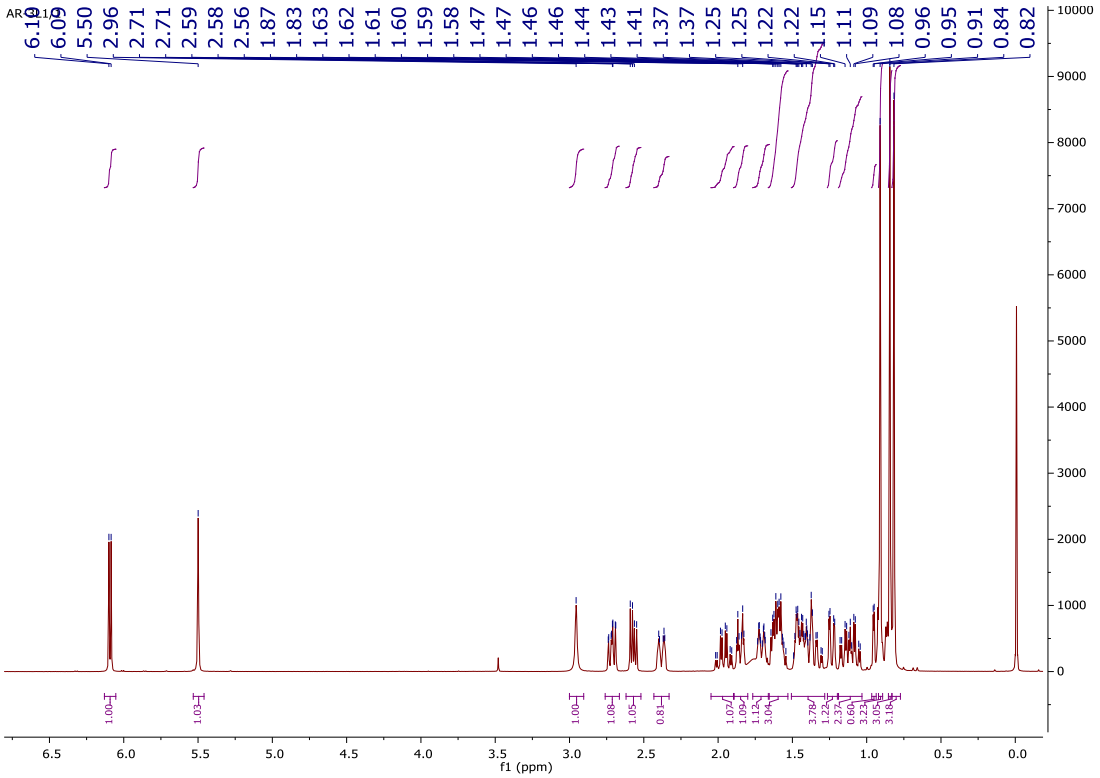
AR66 NOESY spectrum of compound 6 in $CDCl_3$



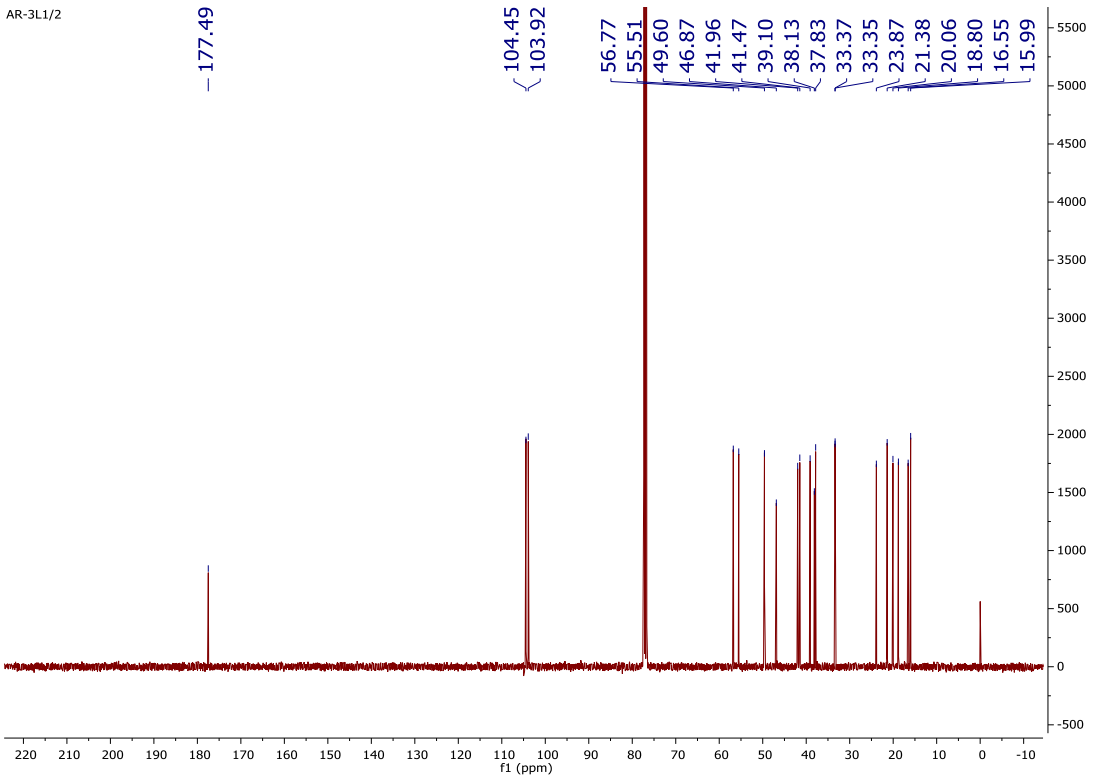
AR67 COSY spectrum of compound 6 in $CDCl_3$



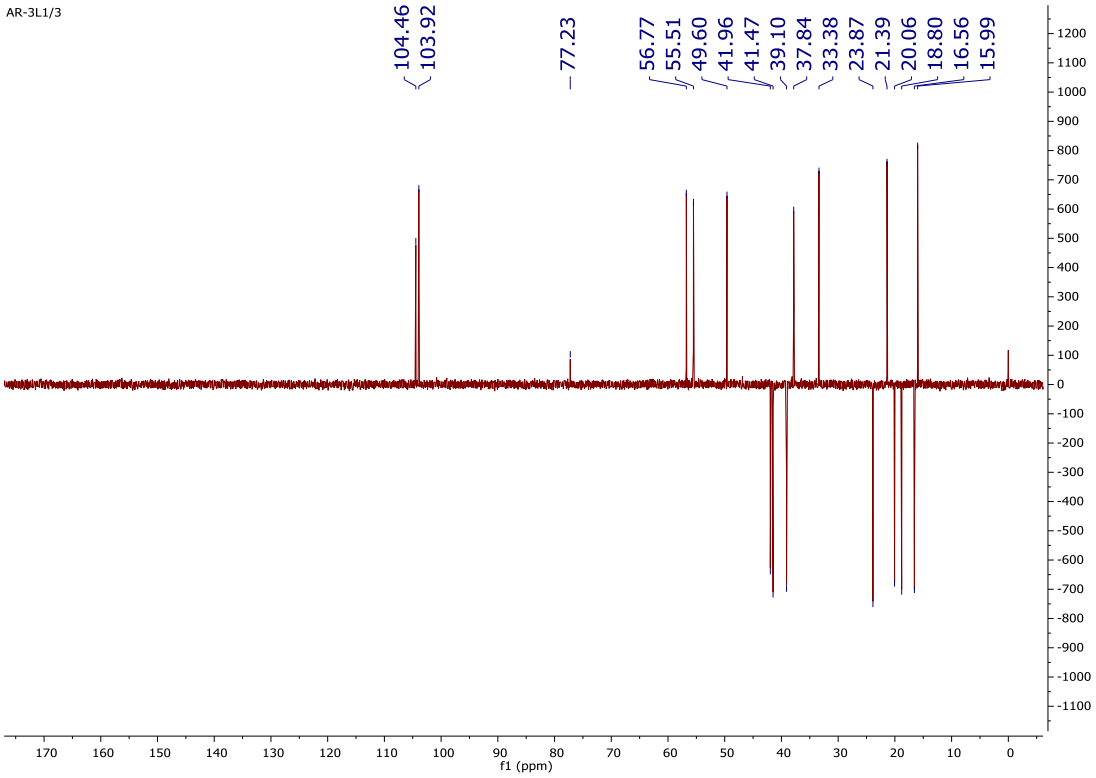
AR71 ^{1}H NMR spectrum of compound 7 in $CDCl_3$



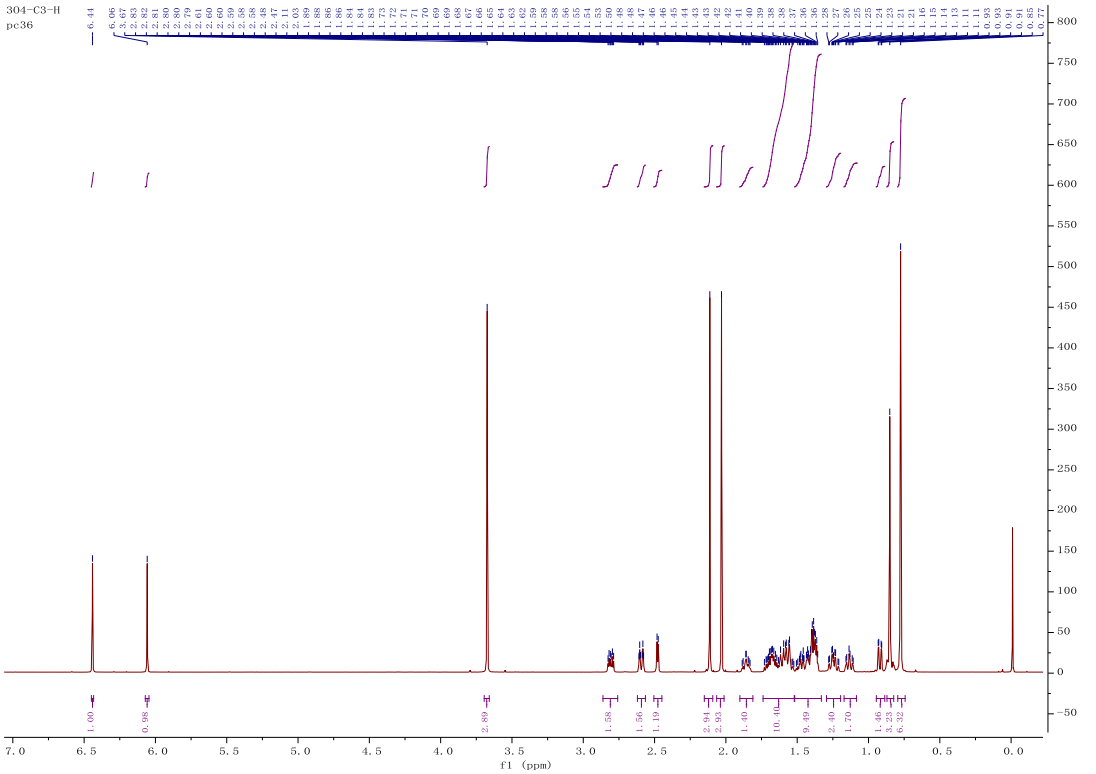
AR72 ^{13}C NMR spectrum of compound 7 in $CDCl_3$



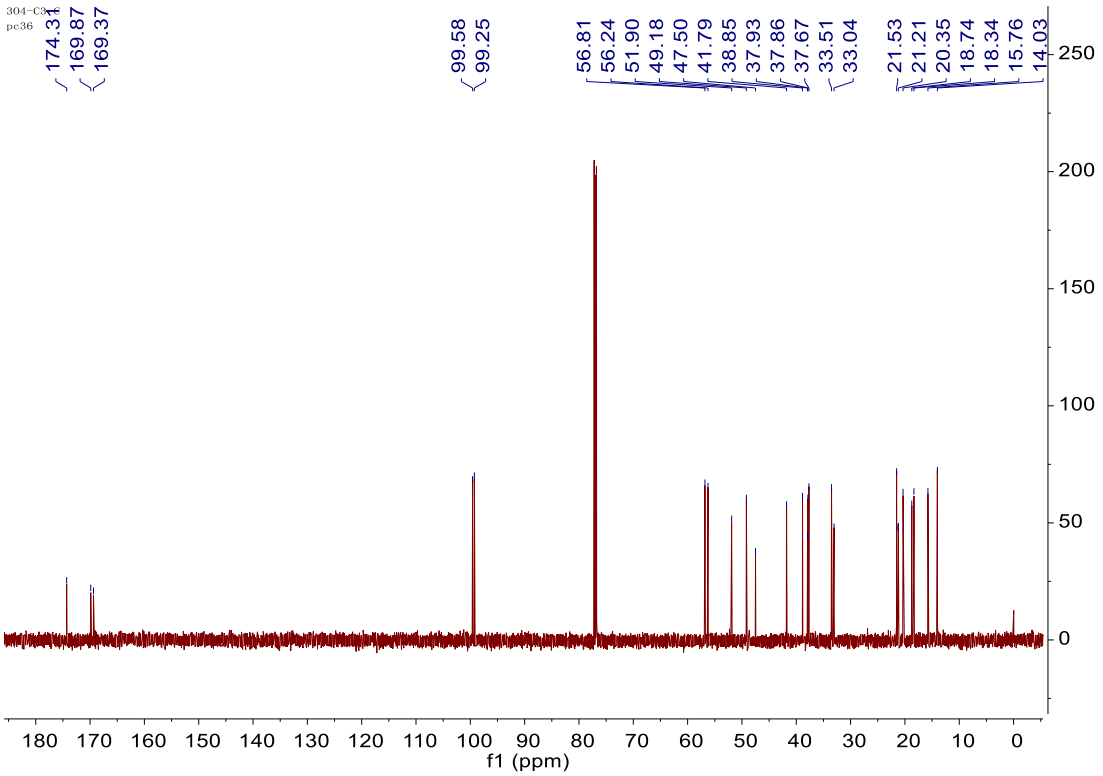
AR73 DEPT spectrum of compound 7 in $CDCl_3$



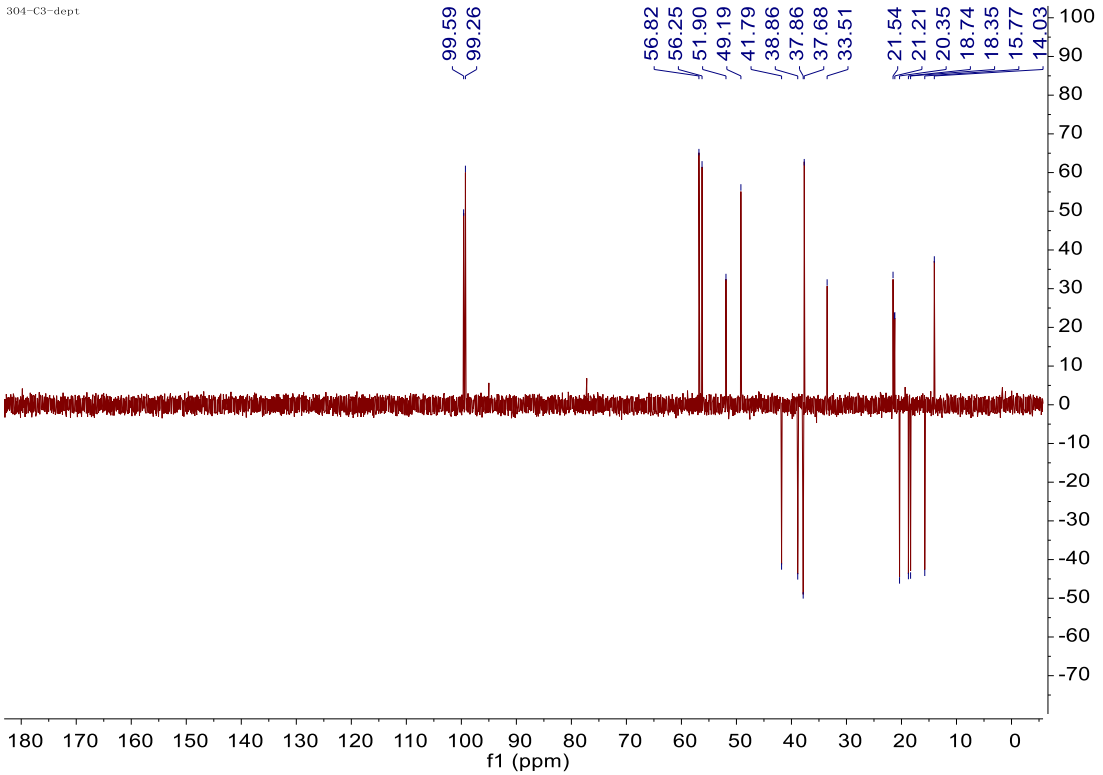
AR81 ^{1}H NMR spectrum of compound 8 in $CDCl_3$



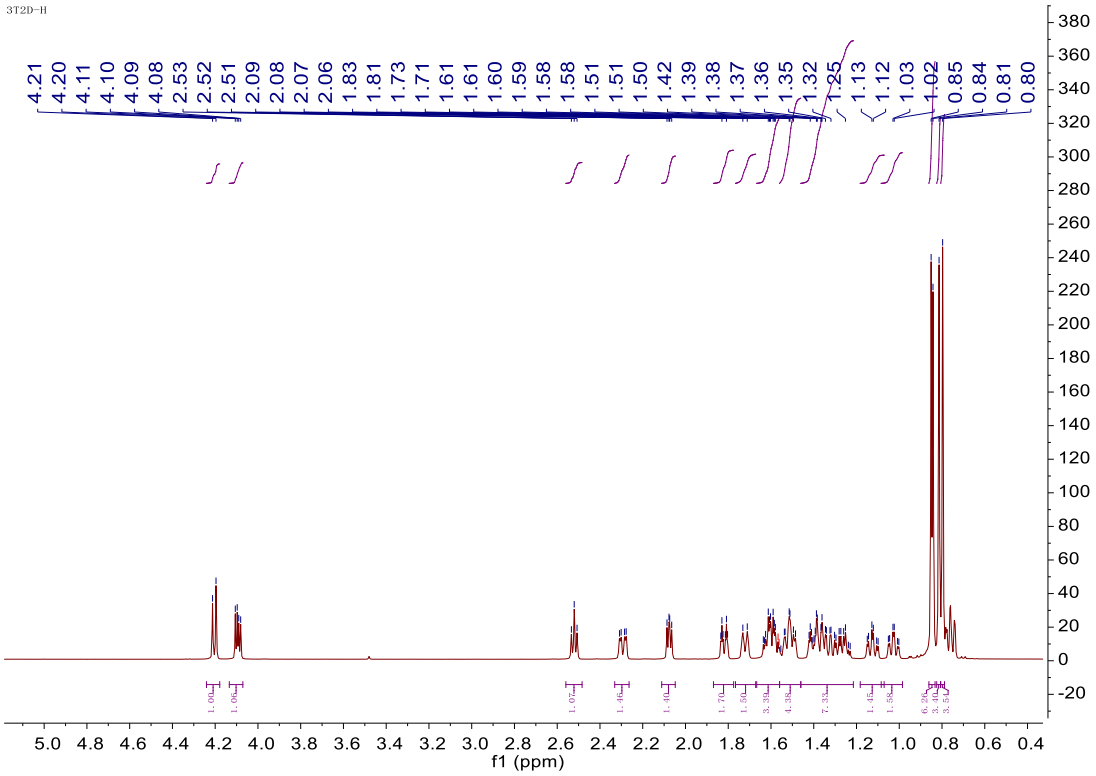
AR82 ^{13}C NMR spectrum of compound 8 in $CDCl_3$



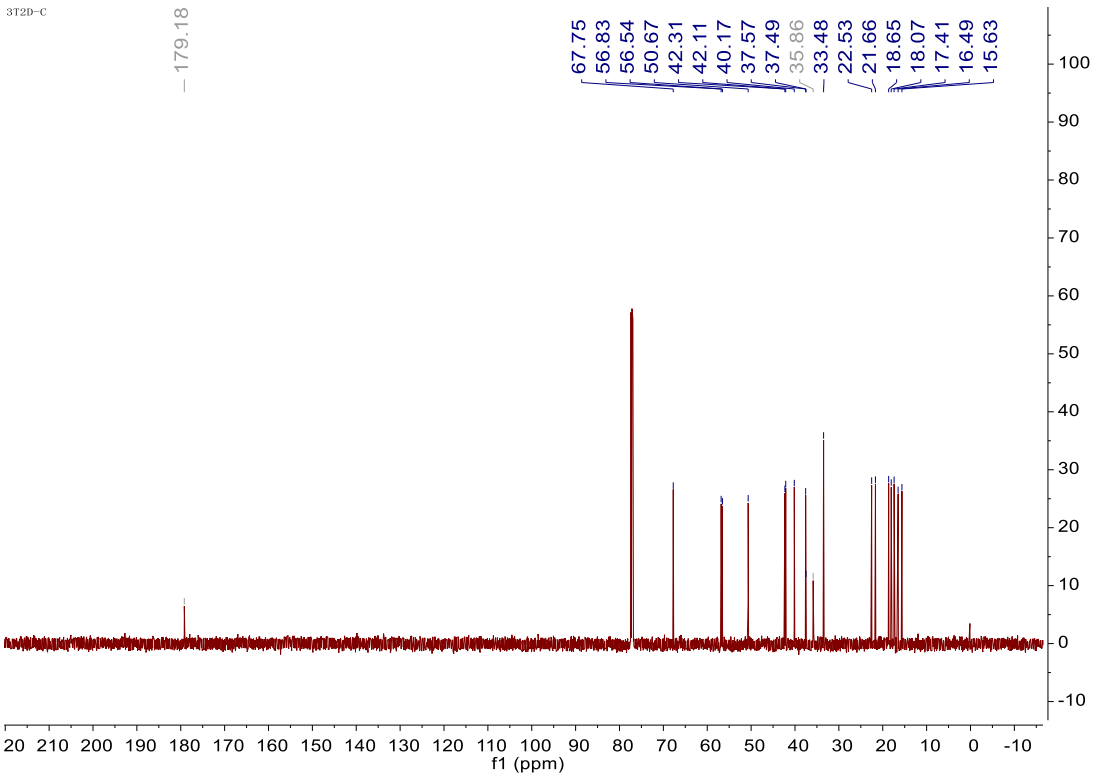
AR83 DEPT spectrum of compound 8 in $CDCl_3$



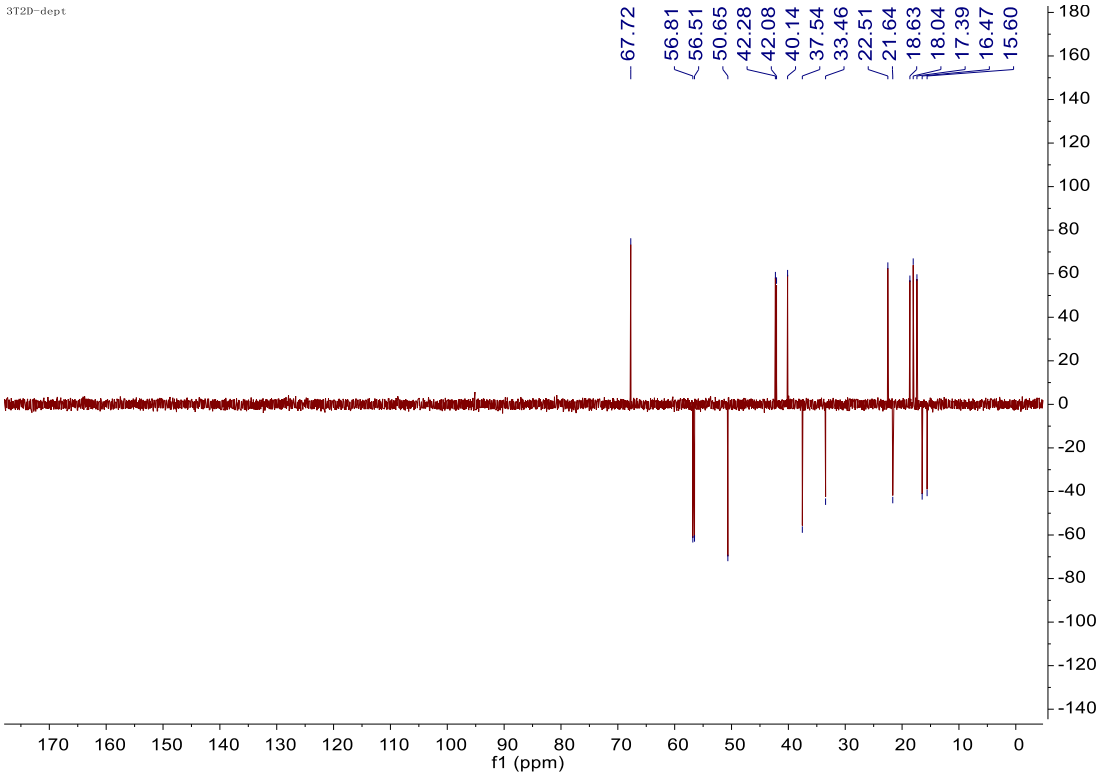
AR91 ^{1}H NMR spectrum of compound 9 in $CDCl_3$



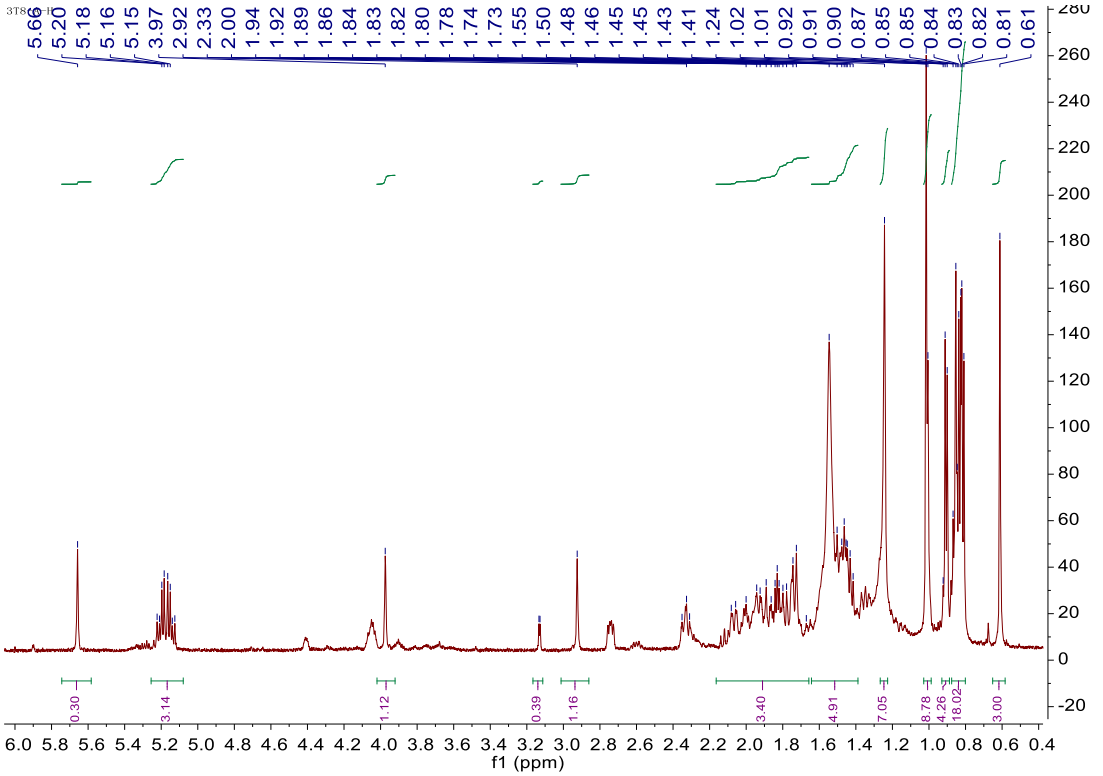
AR92 ^{13}C NMR spectrum of compound 9 in $CDCl_3$



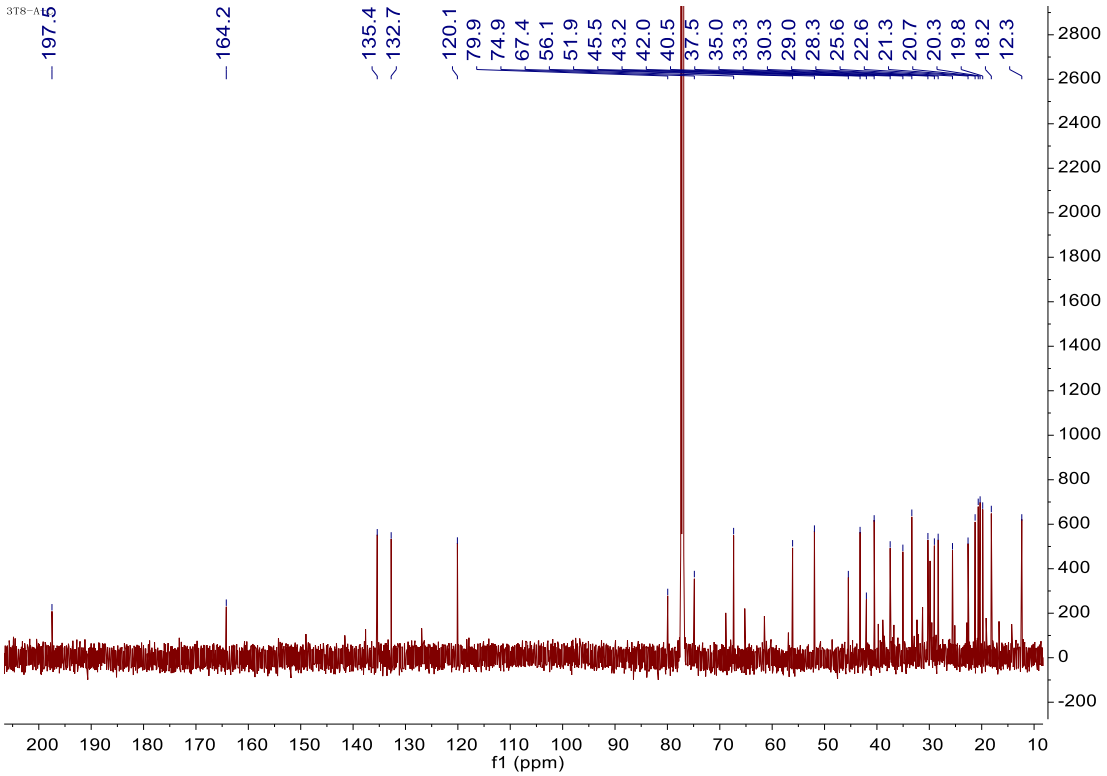
AR93 DEPT spectrum of compound 9 in $CDCl_3$



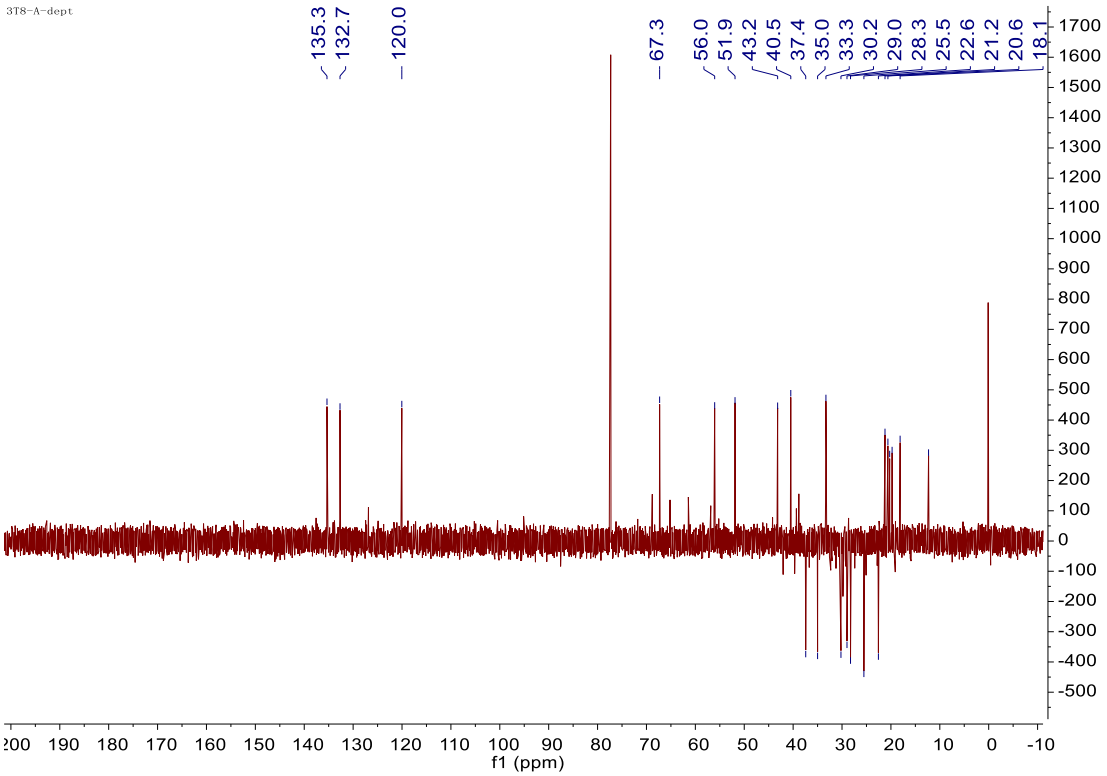
AR101 ^{1}H NMR spectrum of compound 10 in $CDCl_3$



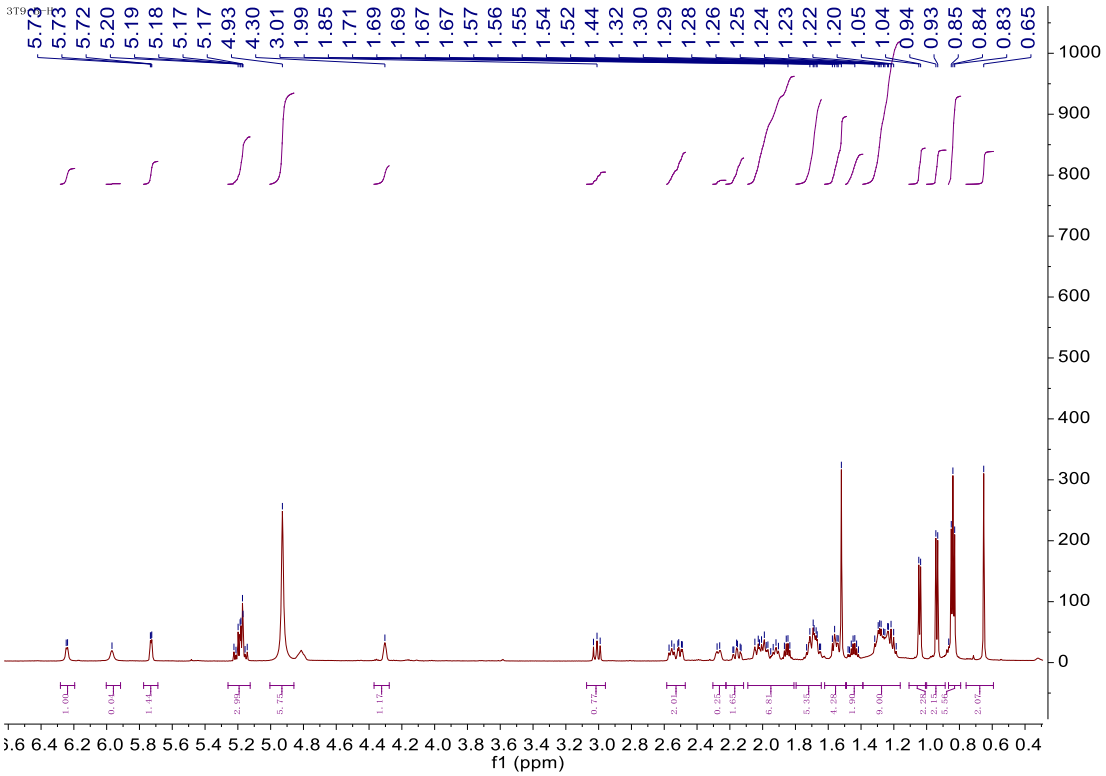
AR102 ^{13}C NMR spectrum of compound 10 in $CDCl_3$



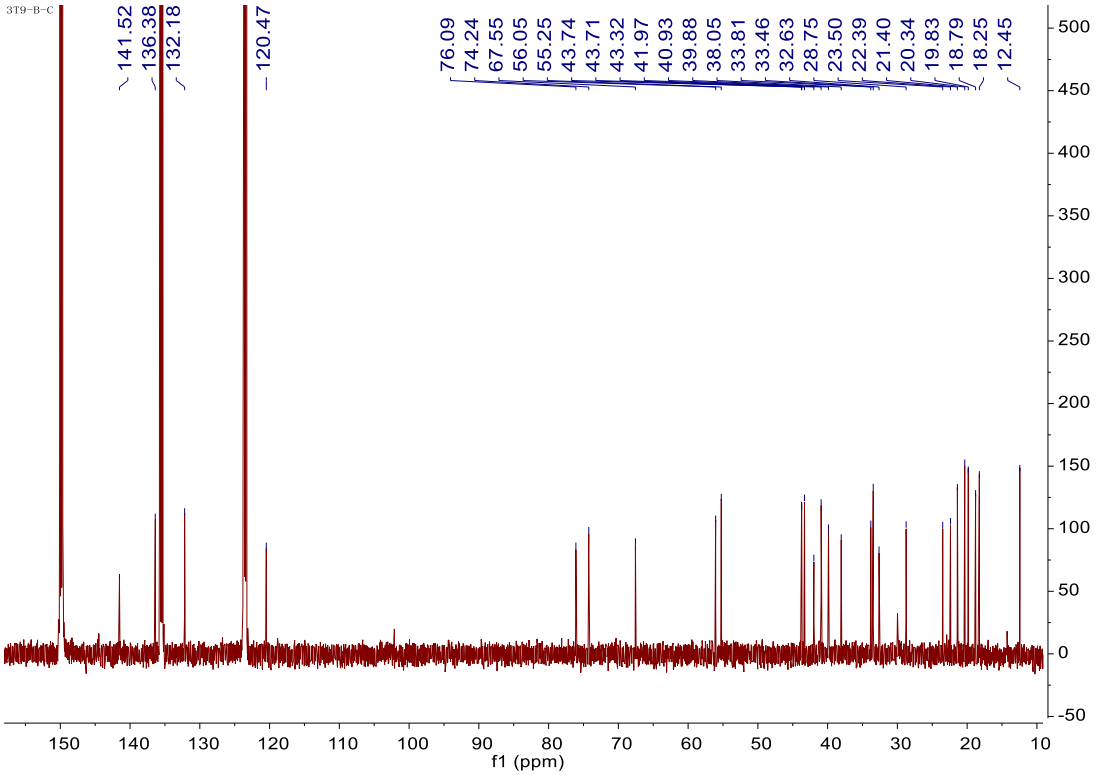
AR103 DEPT spectrum of compound 10 in $CDCl_3$



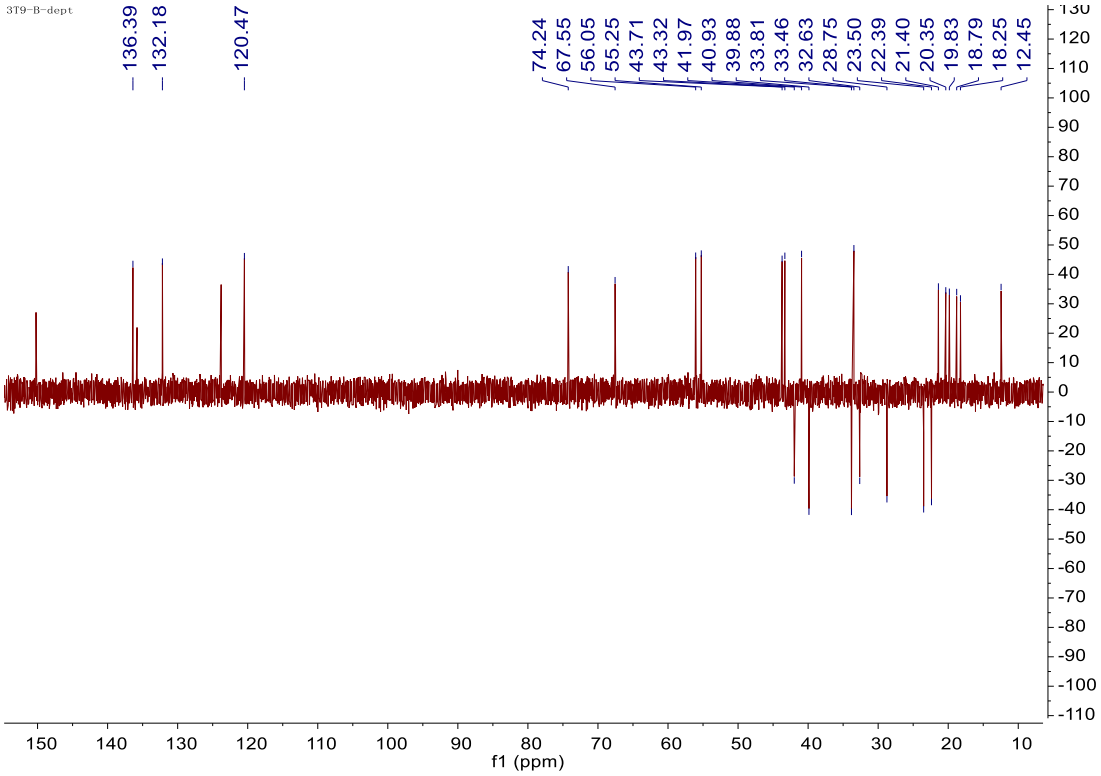
AR111 ^{1}H NMR spectrum of compound 11 in C_6D_5N



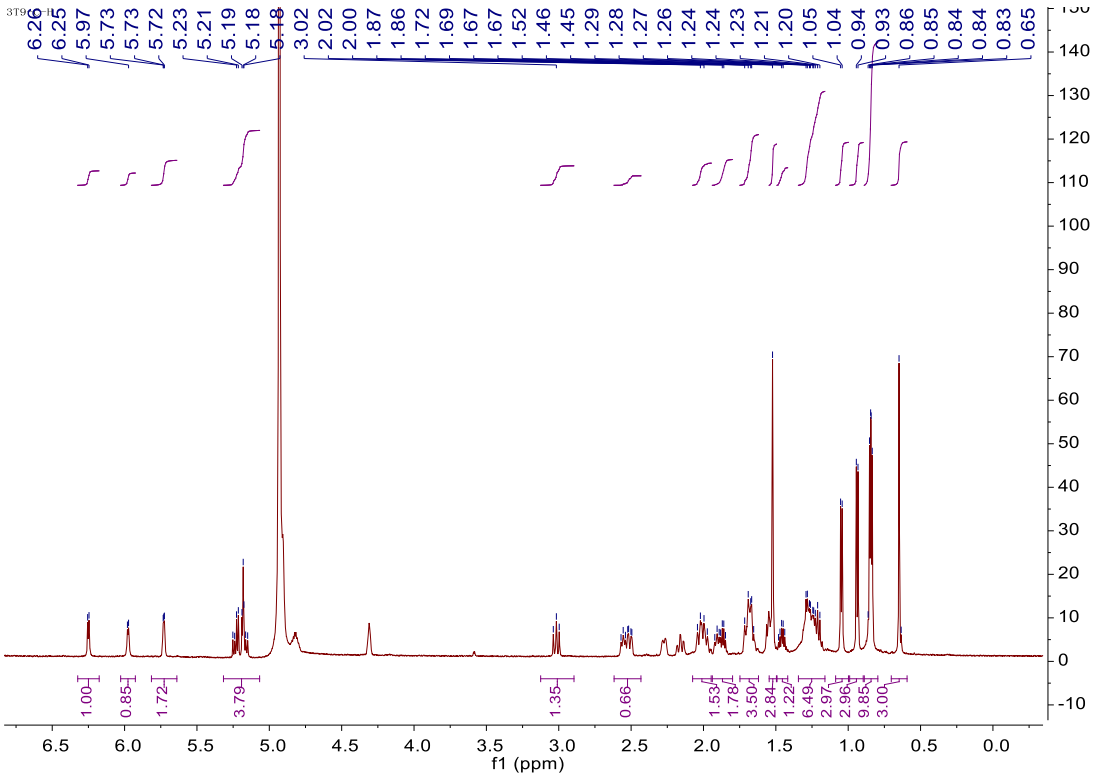
AR112 ^{13}C NMR spectrum of compound 11 in C_6D_5N



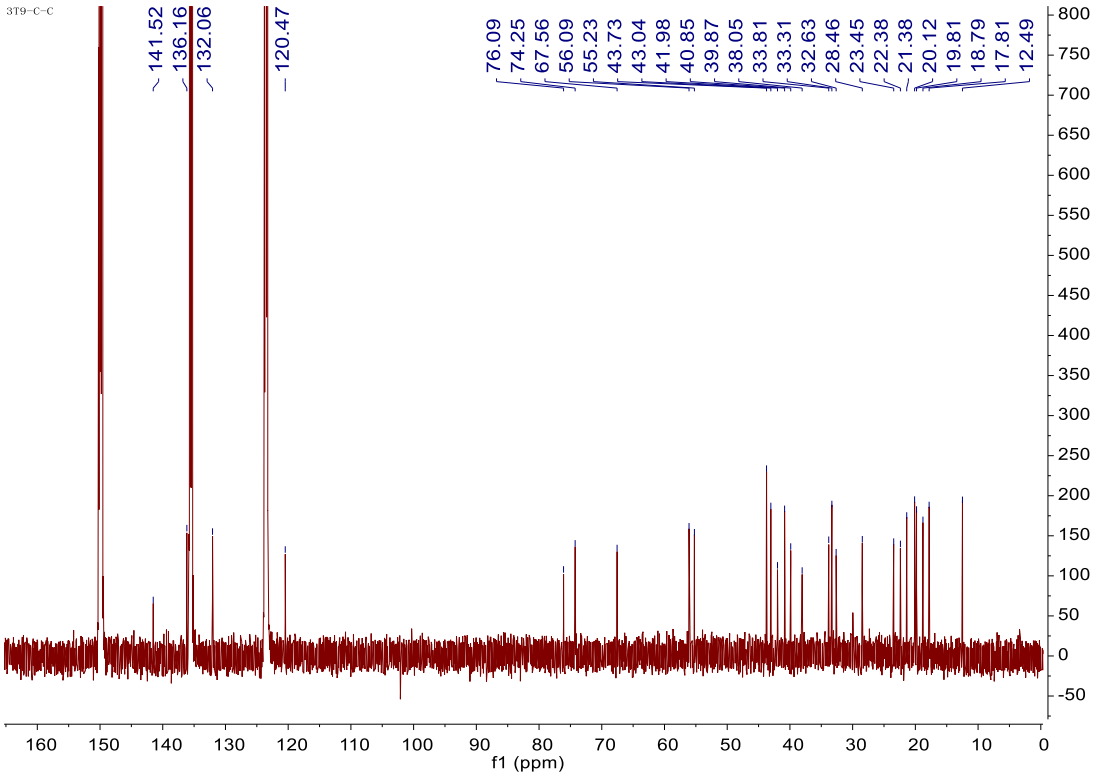
AR113 DEPT spectrum of compound 11 in C_6D_5N



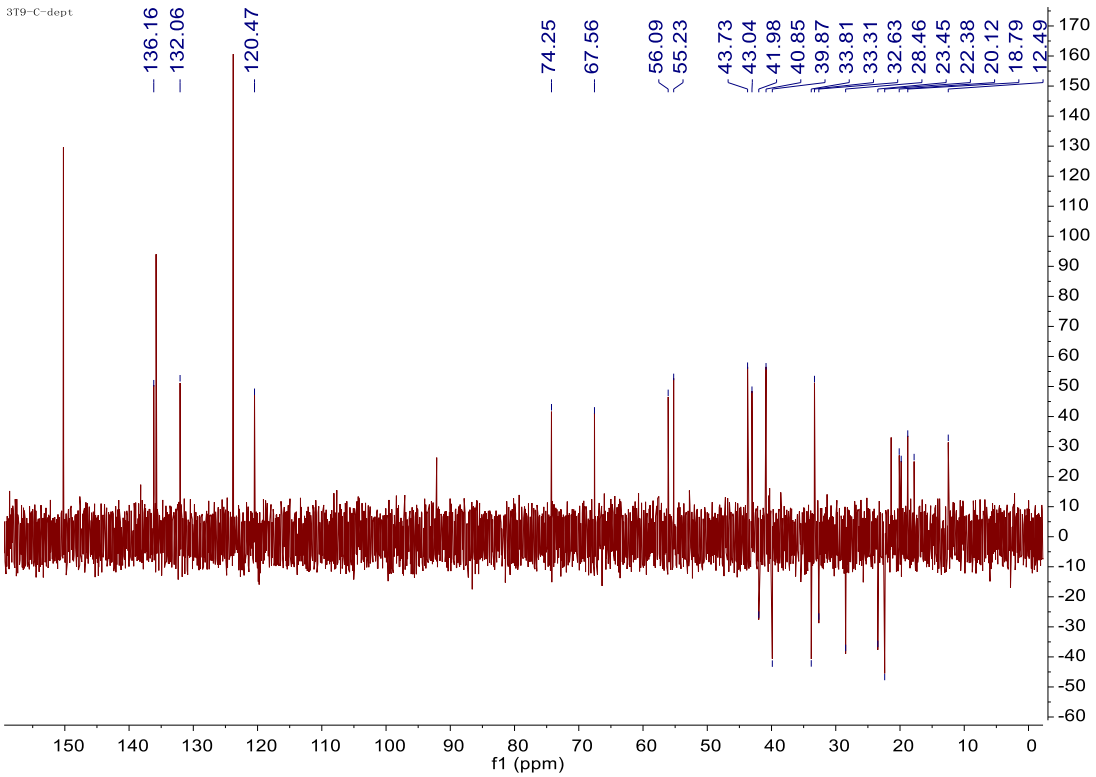
AR121 ^1H NMR spectrum of compound 12 in C_6D_5N



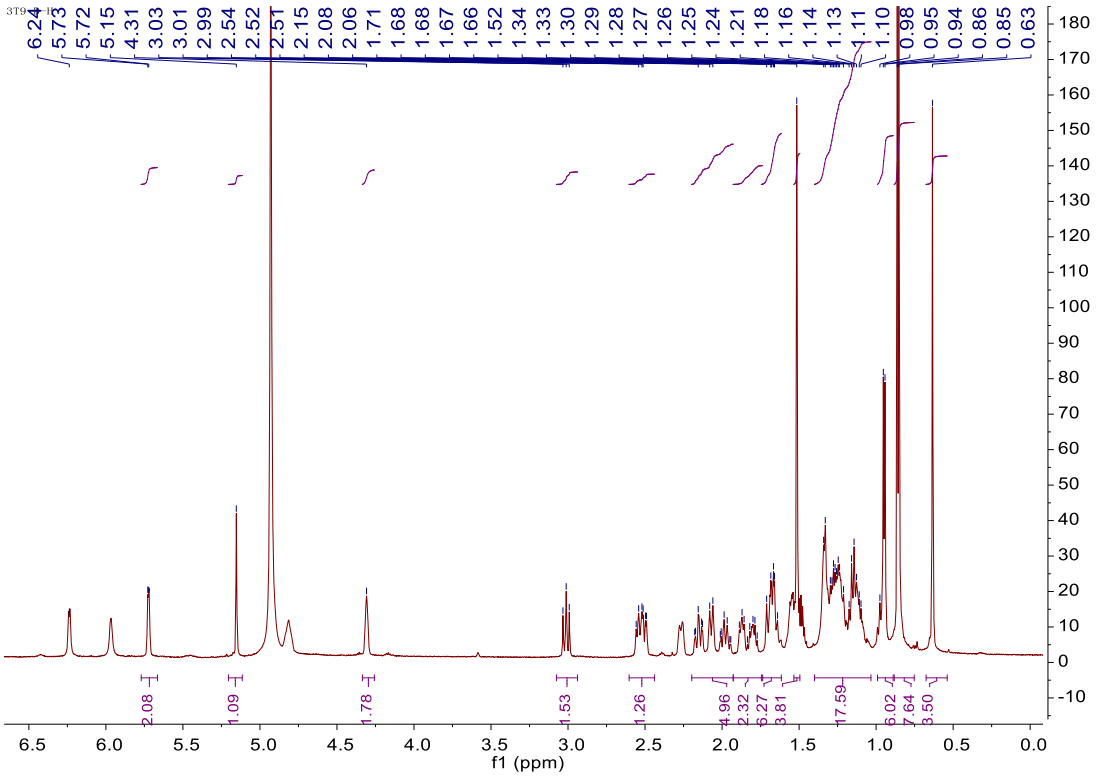
AR122 ^{13}C NMR spectrum of compound 12 in C_6D_5N



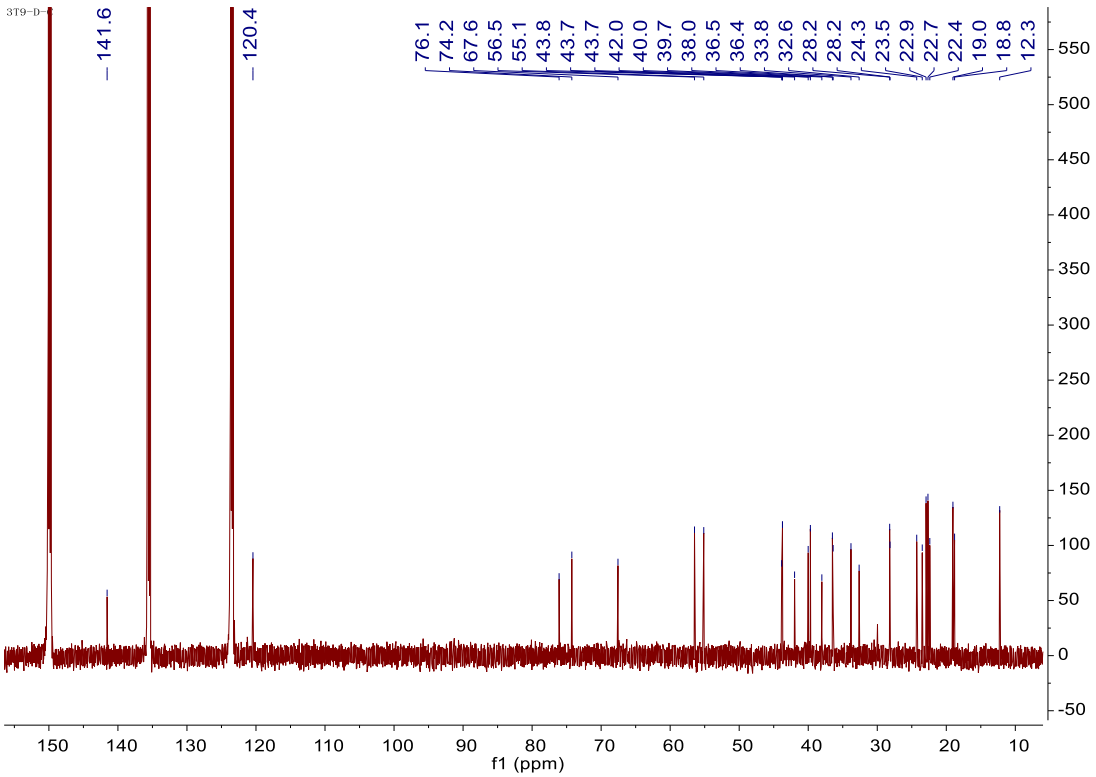
AR123 DEPT spectrum of compound 12 in C_6D_5N



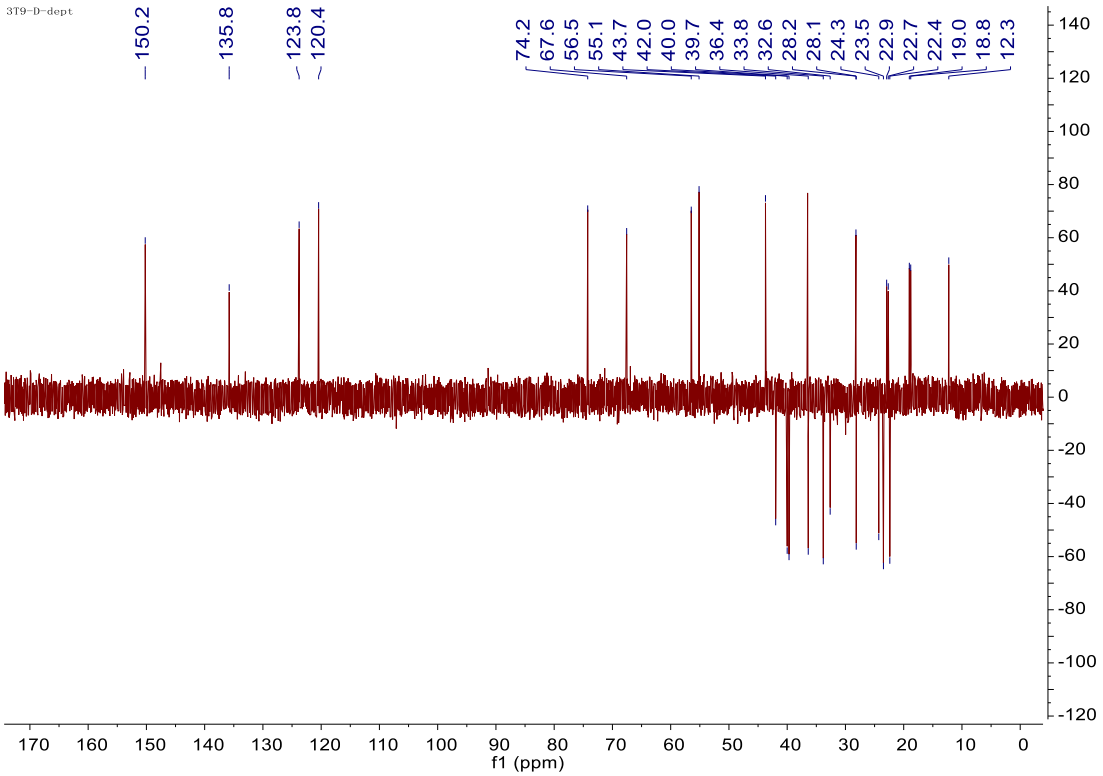
AR131 ^{1}H NMR spectrum of compound 13 in C_6D_5N



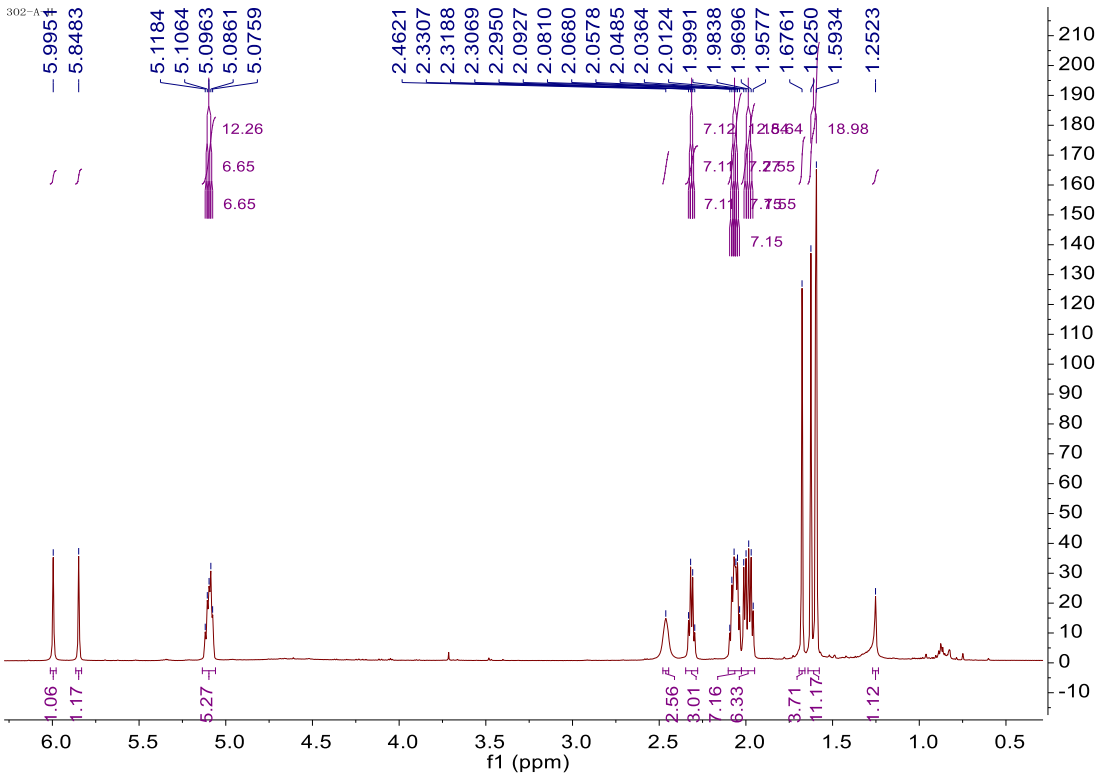
AR132 ^{13}C NMR spectrum of compound 13 in C_6D_5N



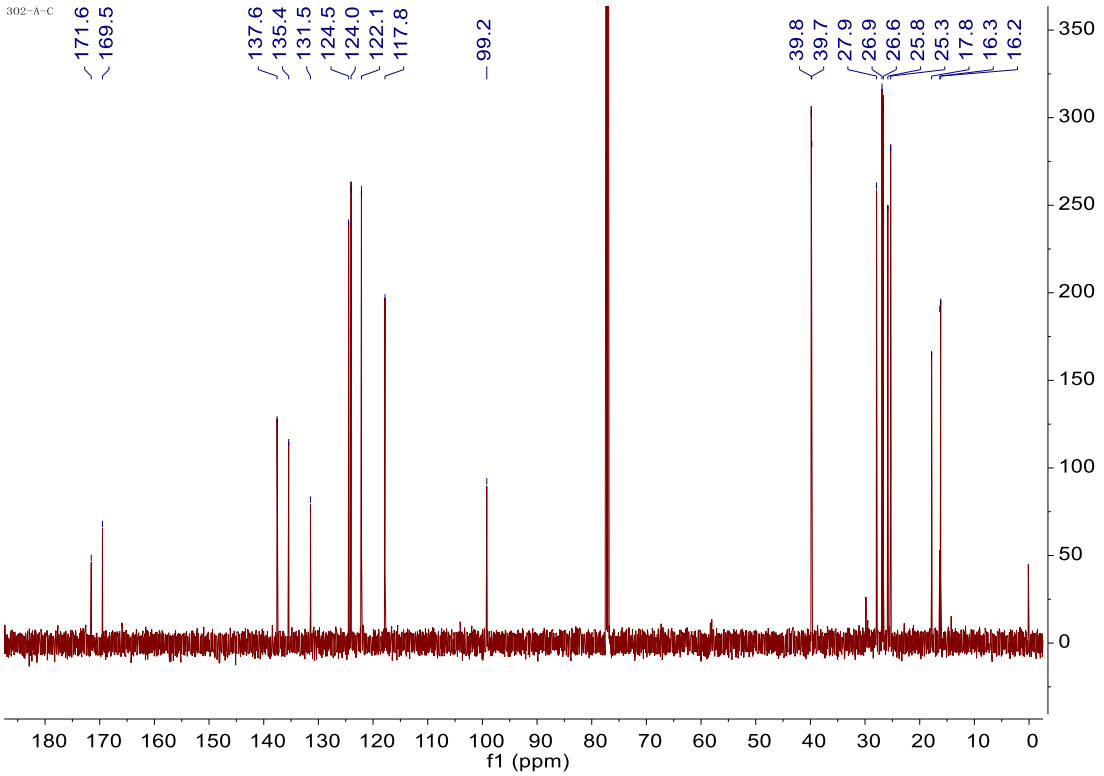
AR133 DEPT spectrum of compound 13 in C_6D_5N



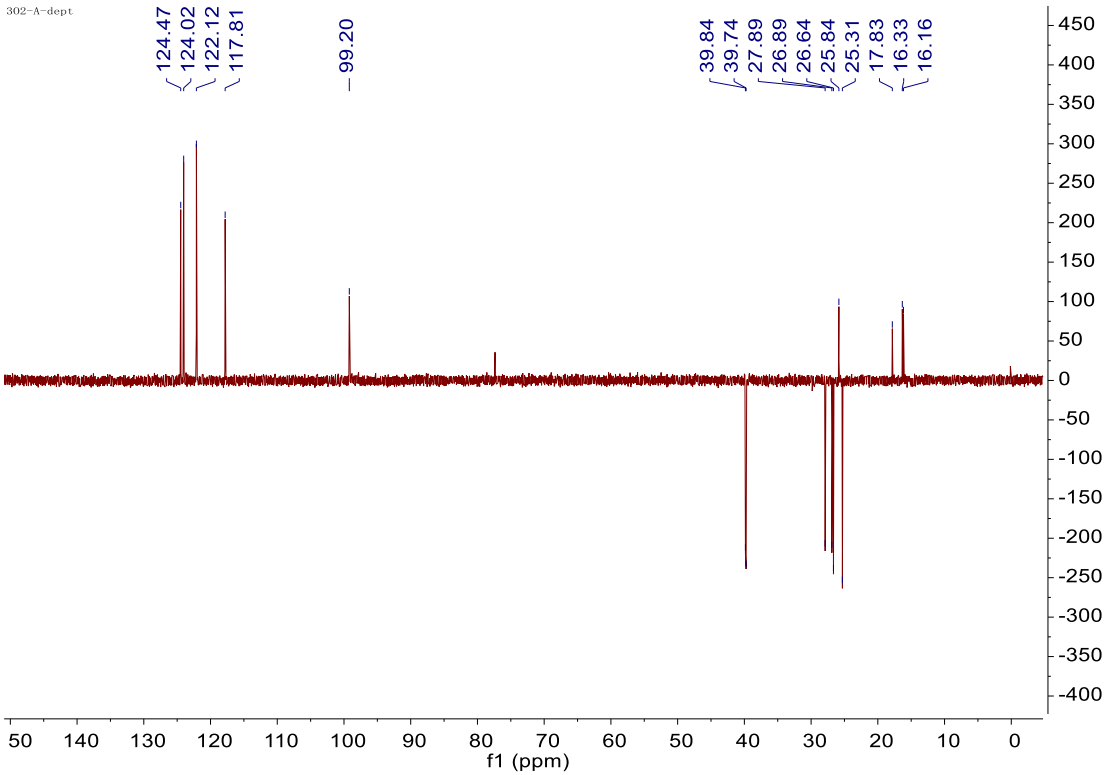
AR141 ^{1}H NMR spectrum of compound 14 in $CDCl_3$



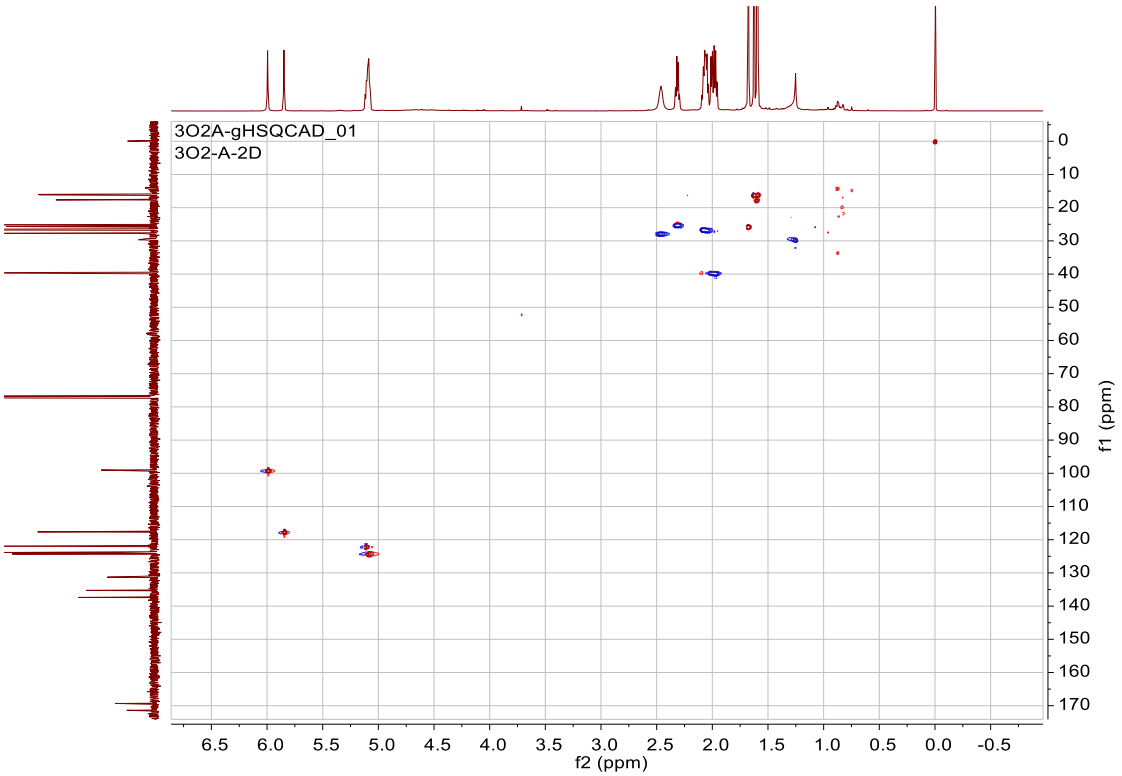
AR142 ^{13}C NMR spectrum of compound 14 in $CDCl_3$



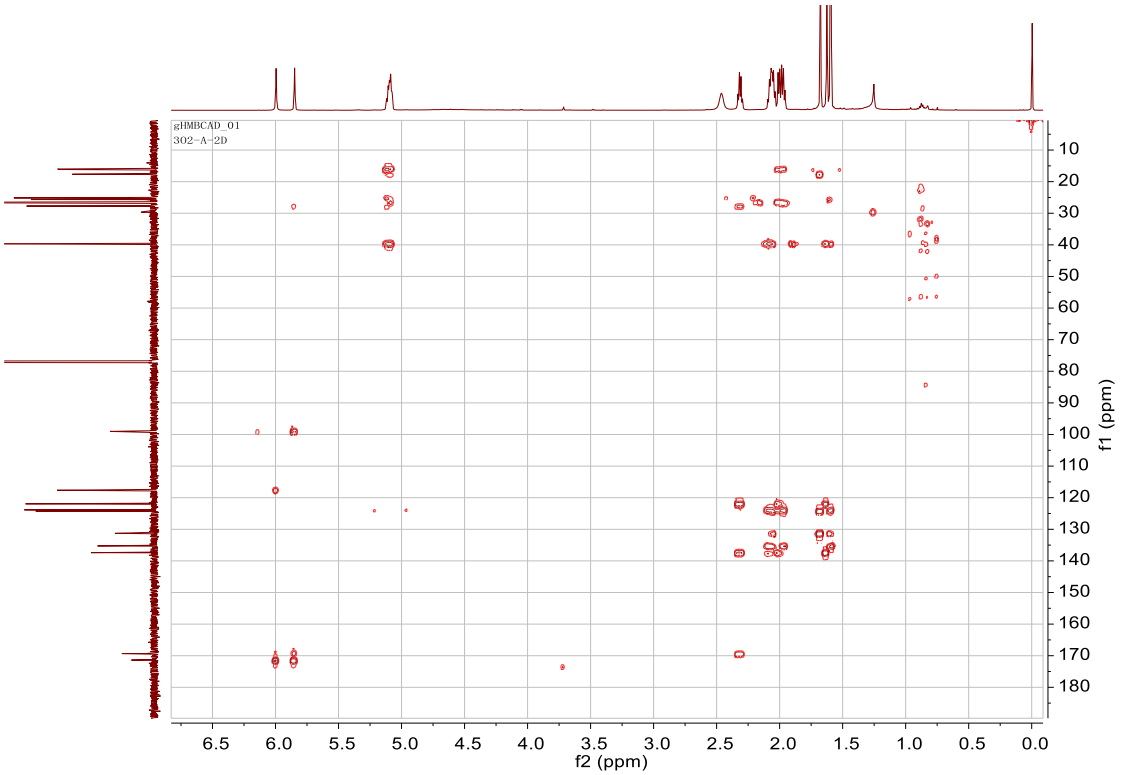
AR143 DEPT spectrum of compound 14 in $CDCl_3$



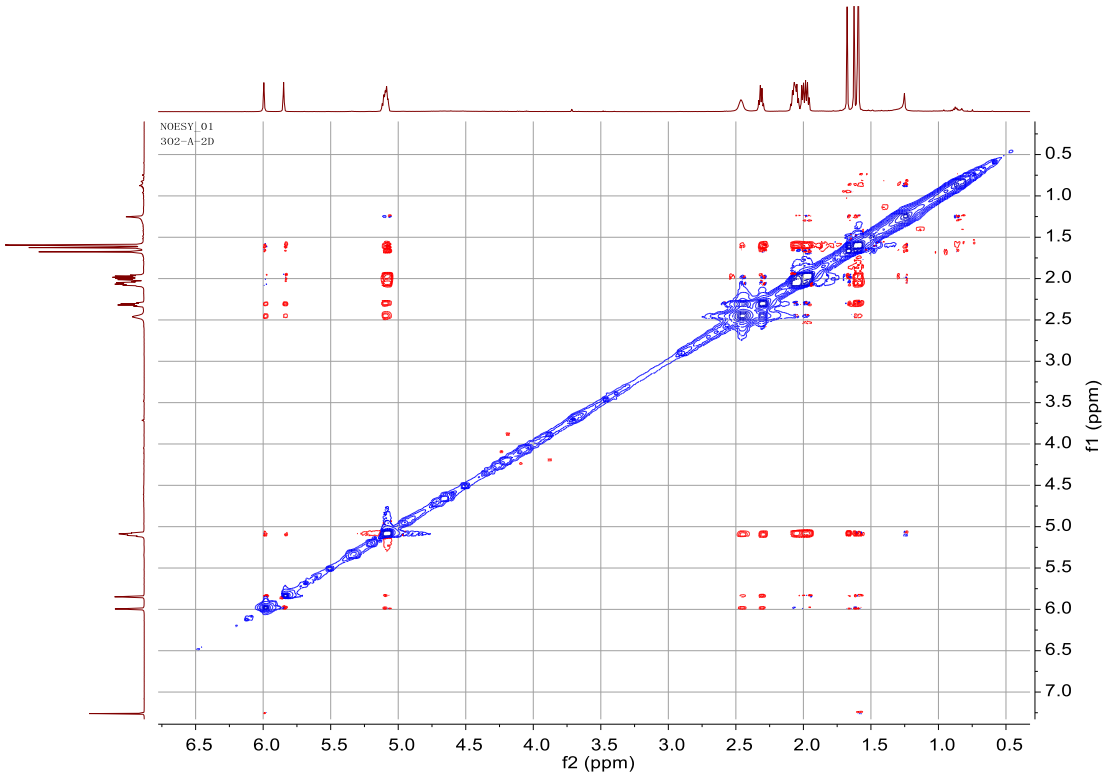
AR144 HSQC spectrum of compound 14 in $CDCl_3$



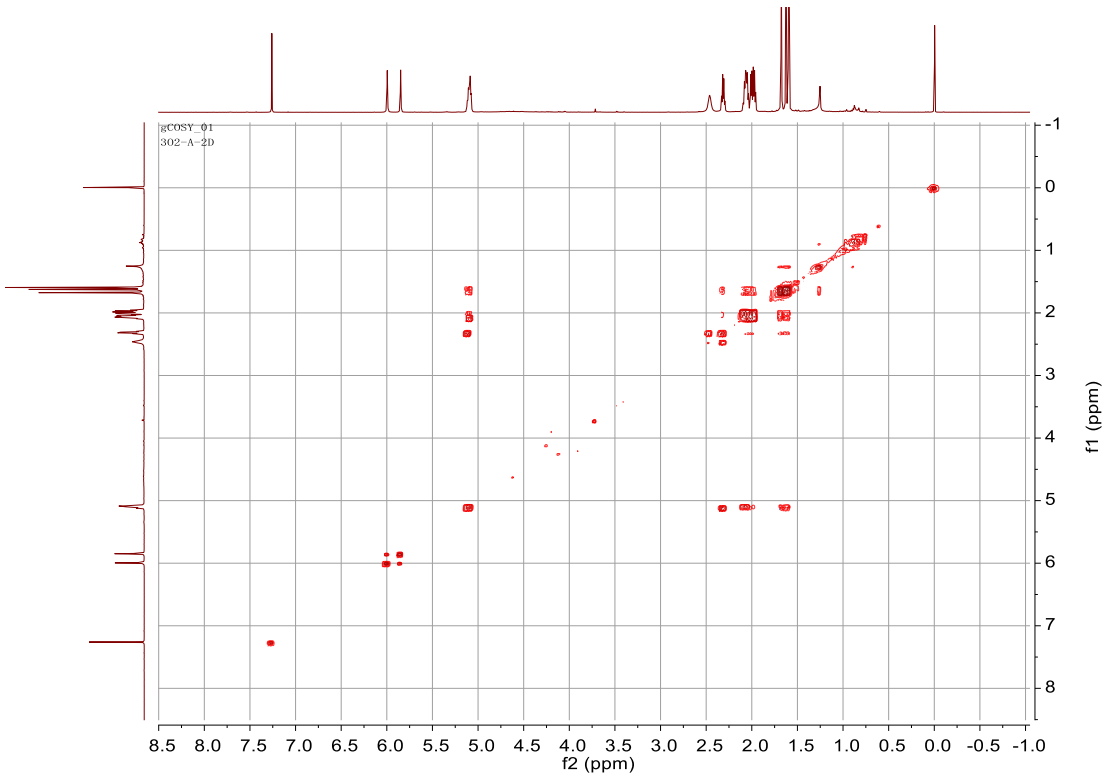
AR145 HMBC spectrum of compound 14 in $CDCl_3$



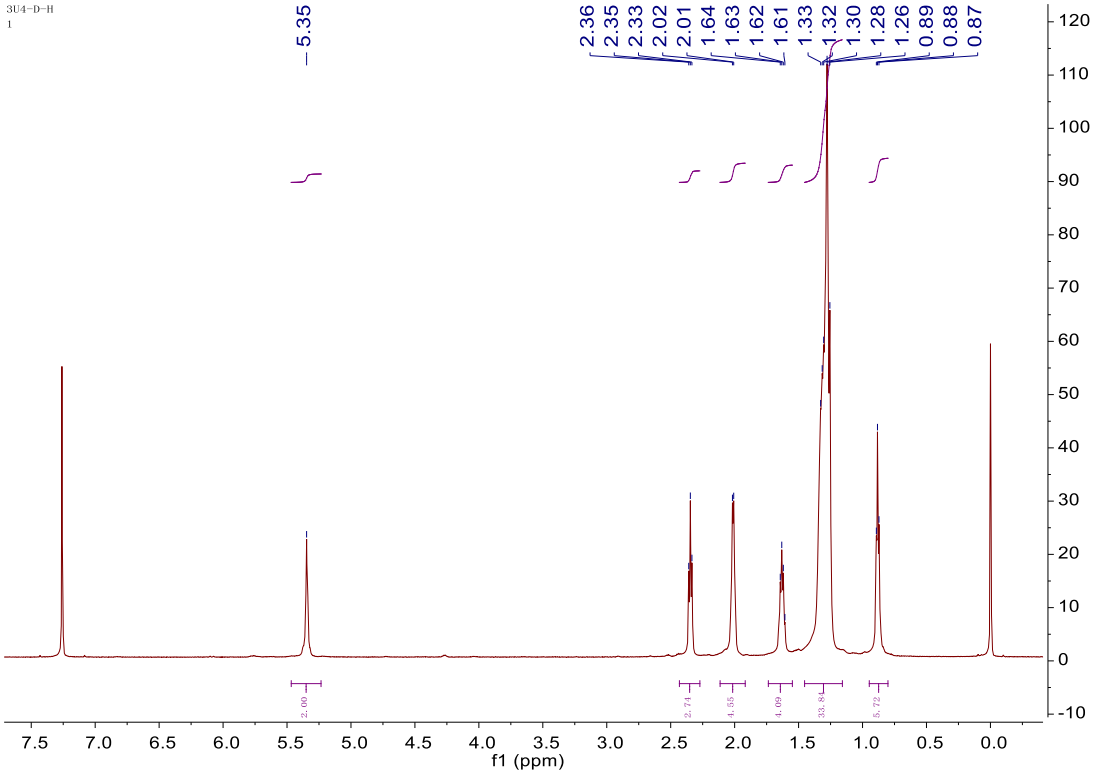
AR146 NOESY spectrum of compound 14 in $CDCl_3$



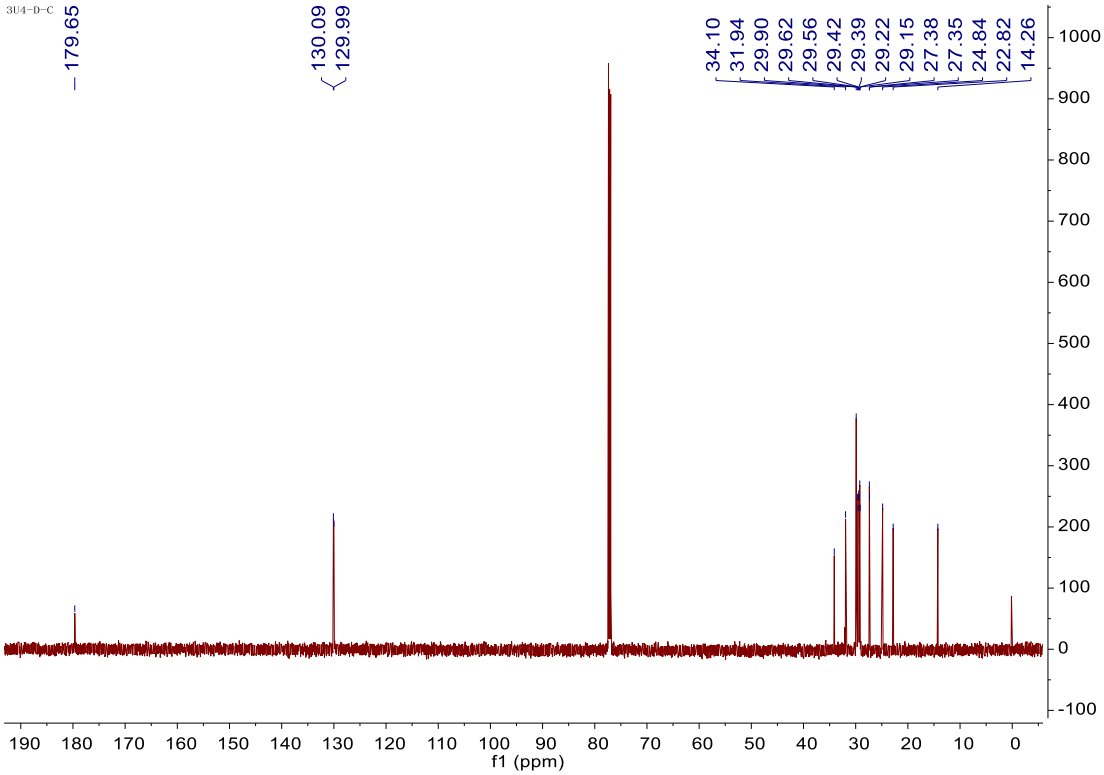
AR147 COSY spectrum of compound 14 in $CDCl_3$



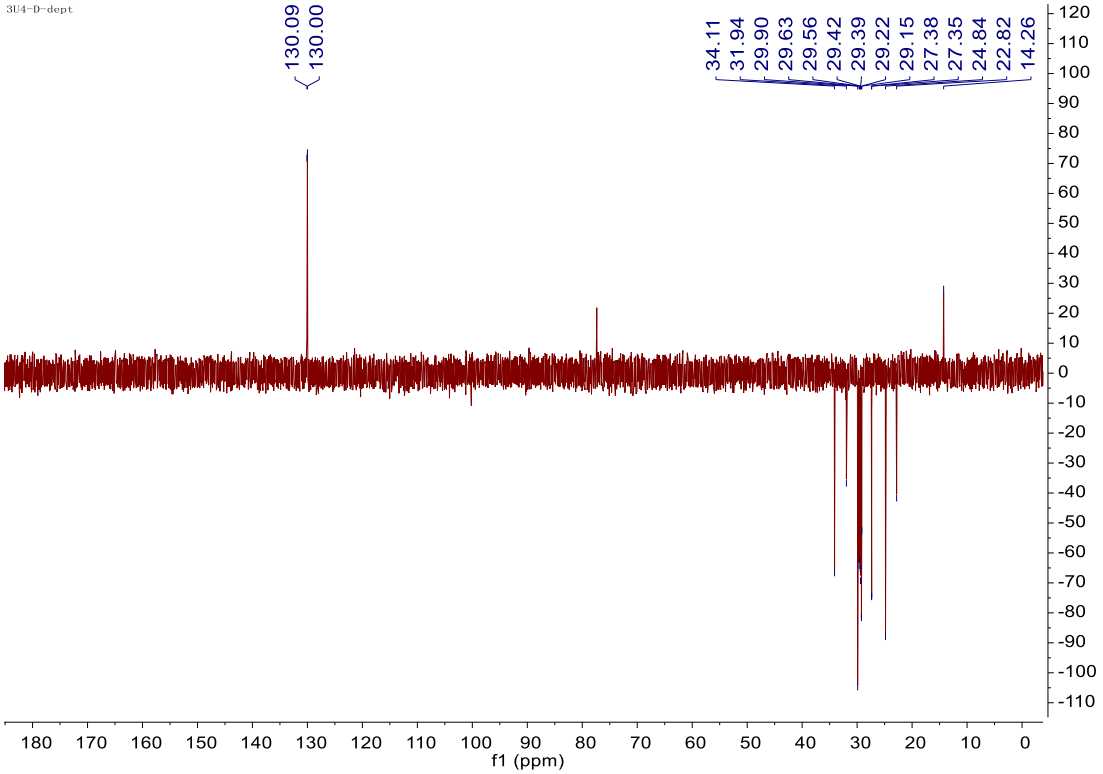
AR151 ^{1}H NMR spectrum of compound 15 in $CDCl_3$



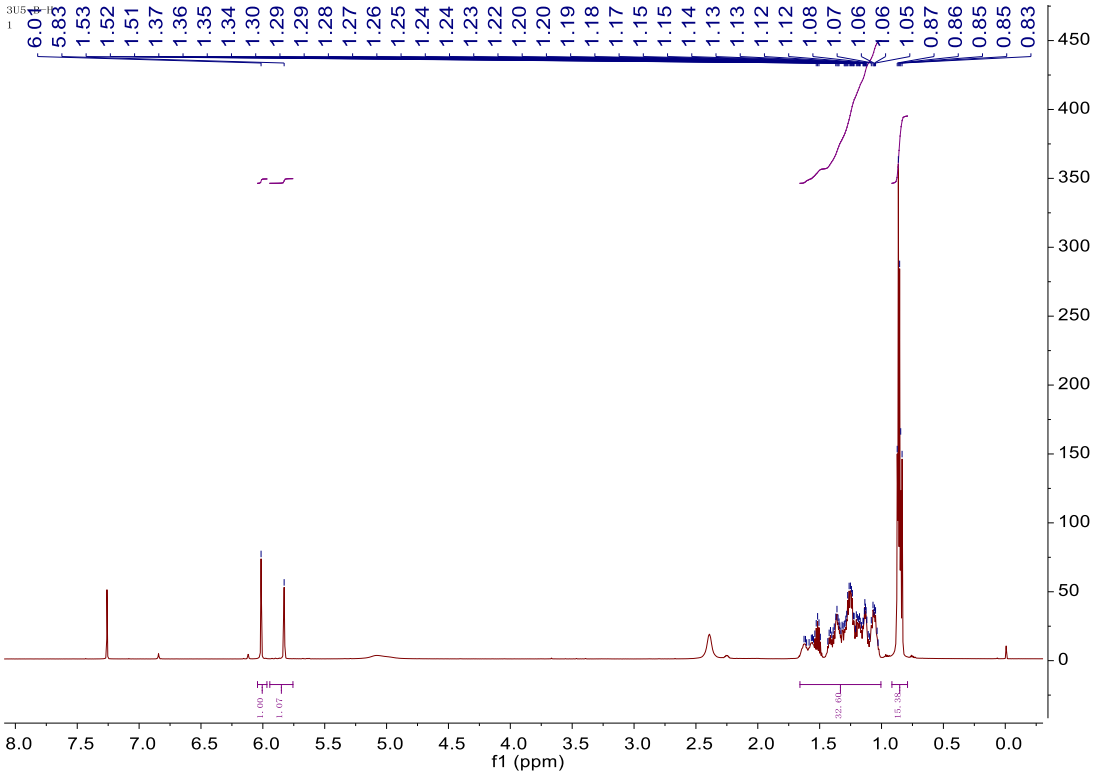
AR152 ^{13}C NMR spectrum of compound 15 in $CDCl_3$



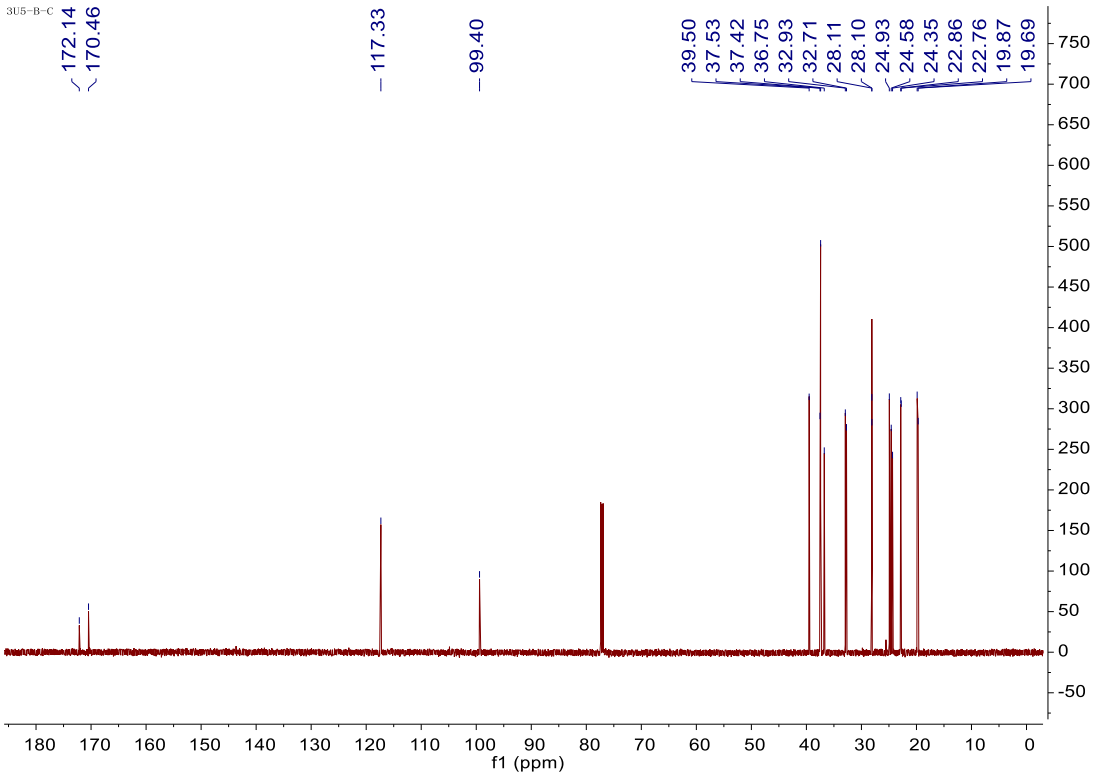
AR153 DEPT spectrum of compound 15 in $CDCl_3$



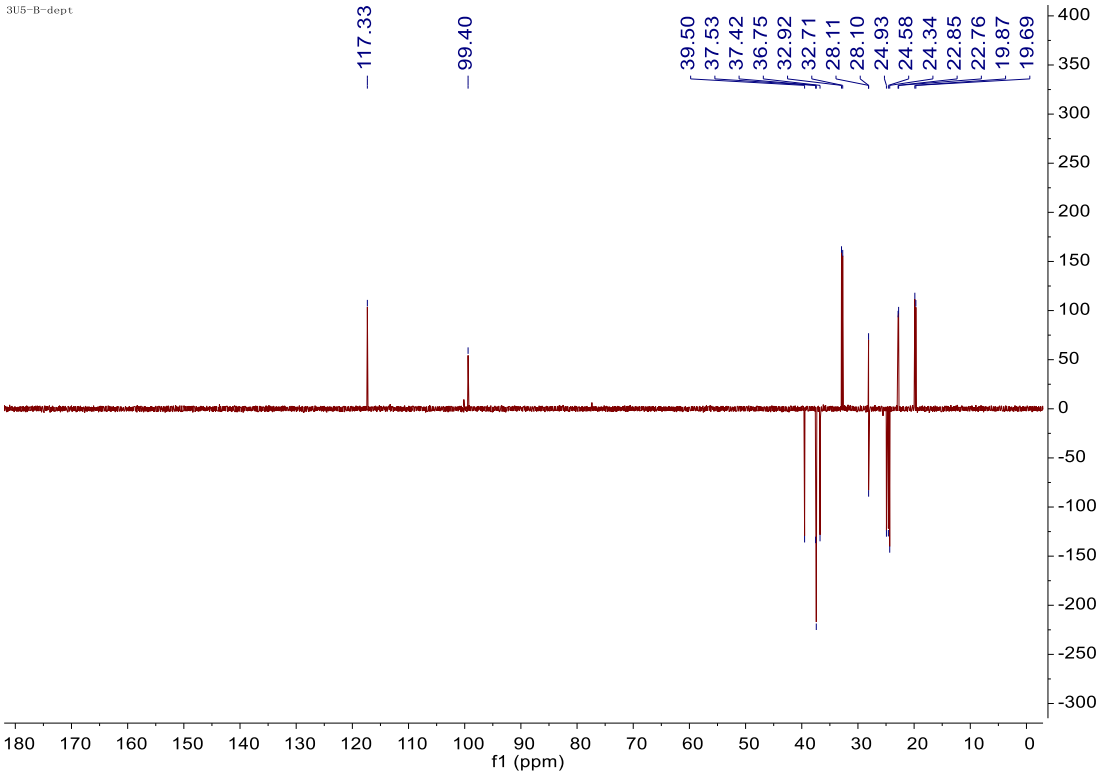
AR161 ^1H NMR spectrum of compound 16 in $CDCl_3$



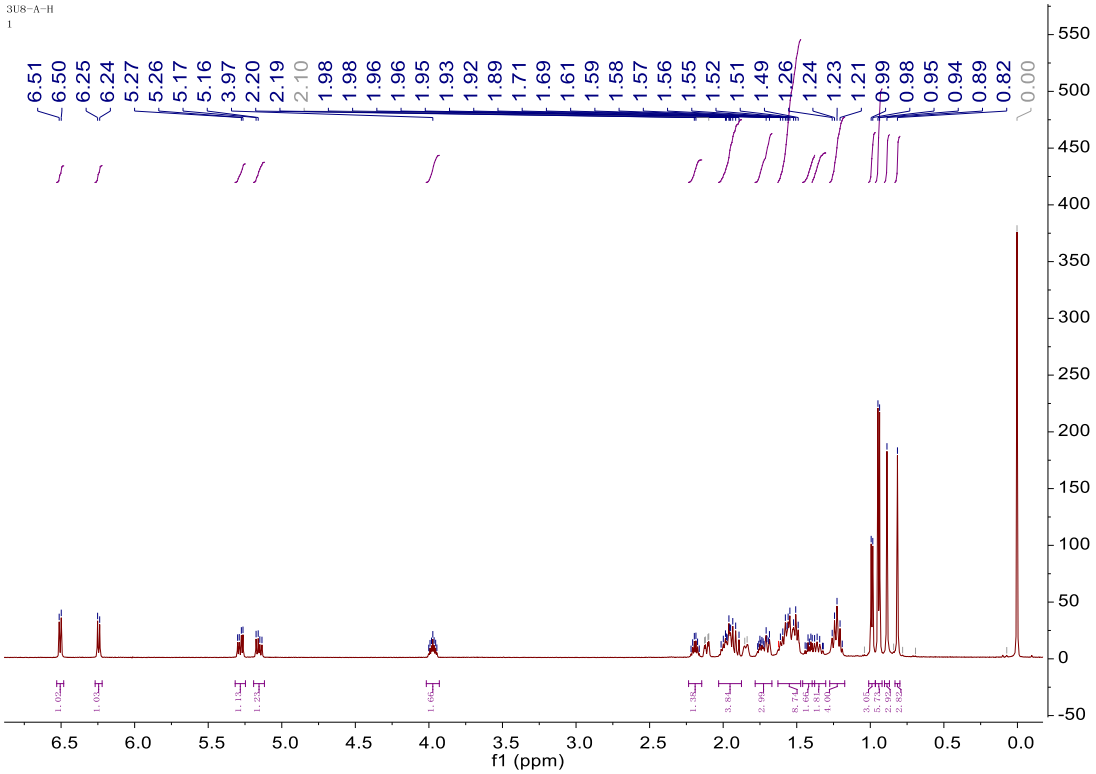
AR162 ^{13}C NMR spectrum of compound 16 in $CDCl_3$



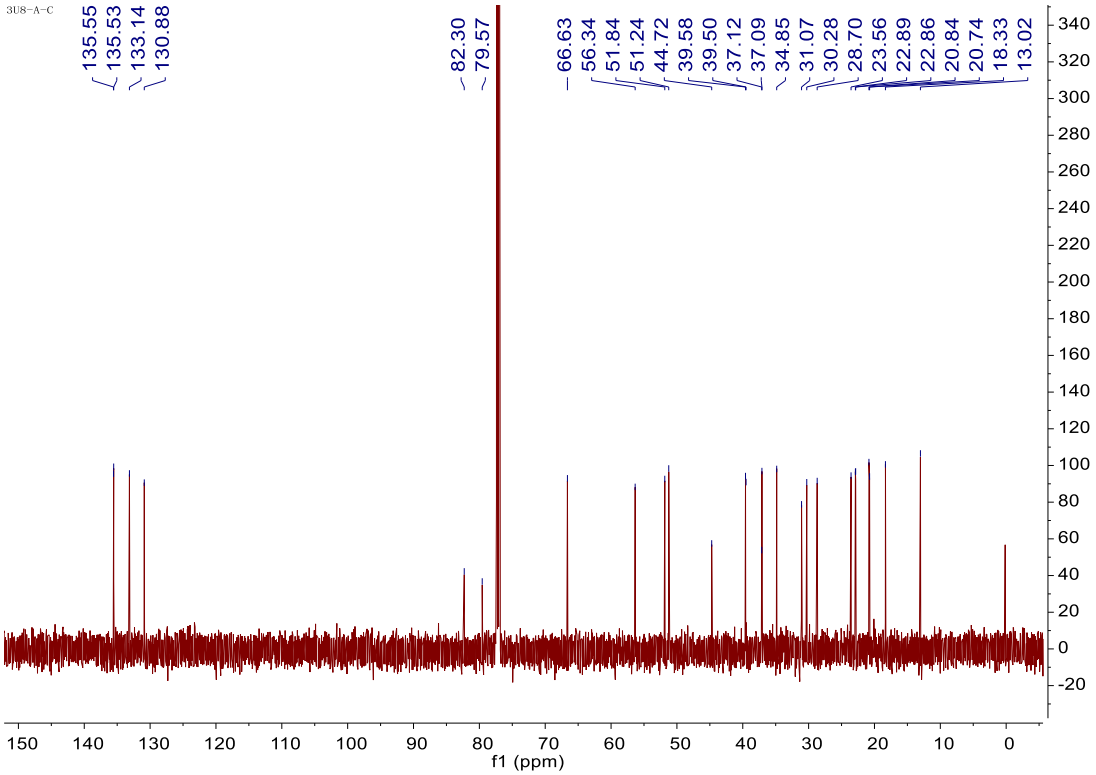
AR163 DEPT spectrum of compound 16 in $CDCl_3$



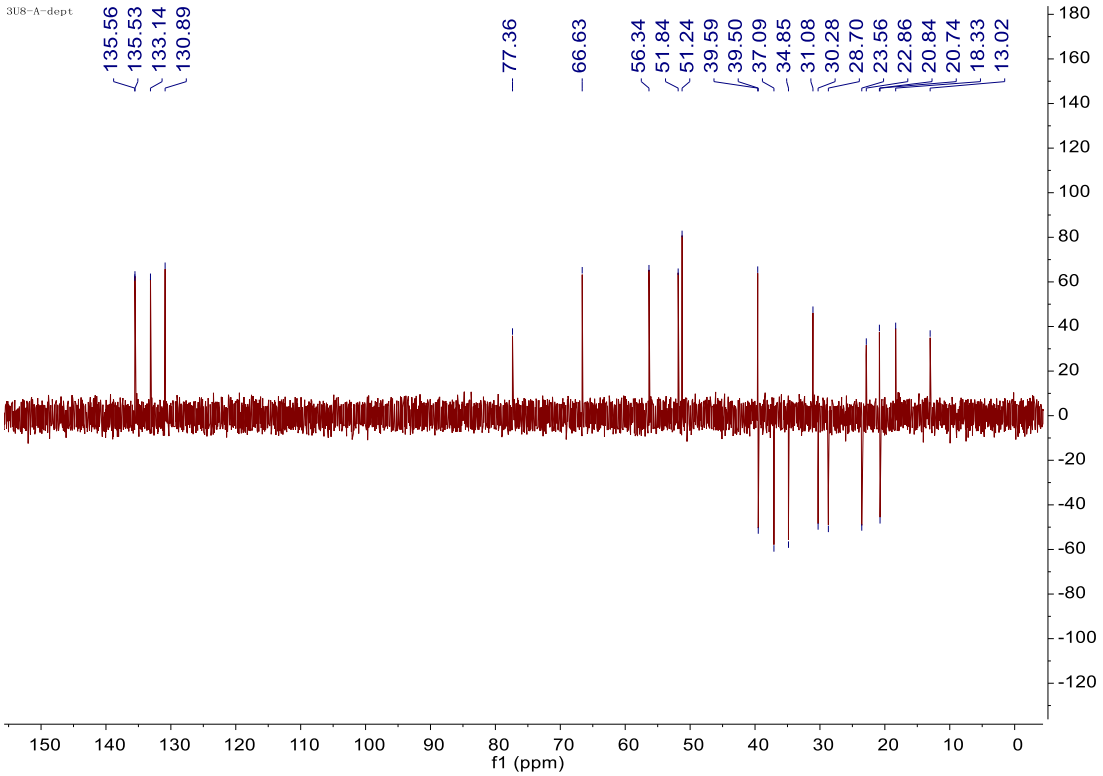
AR171 ^{1}H NMR spectrum of compound 17in $CDCl_3$



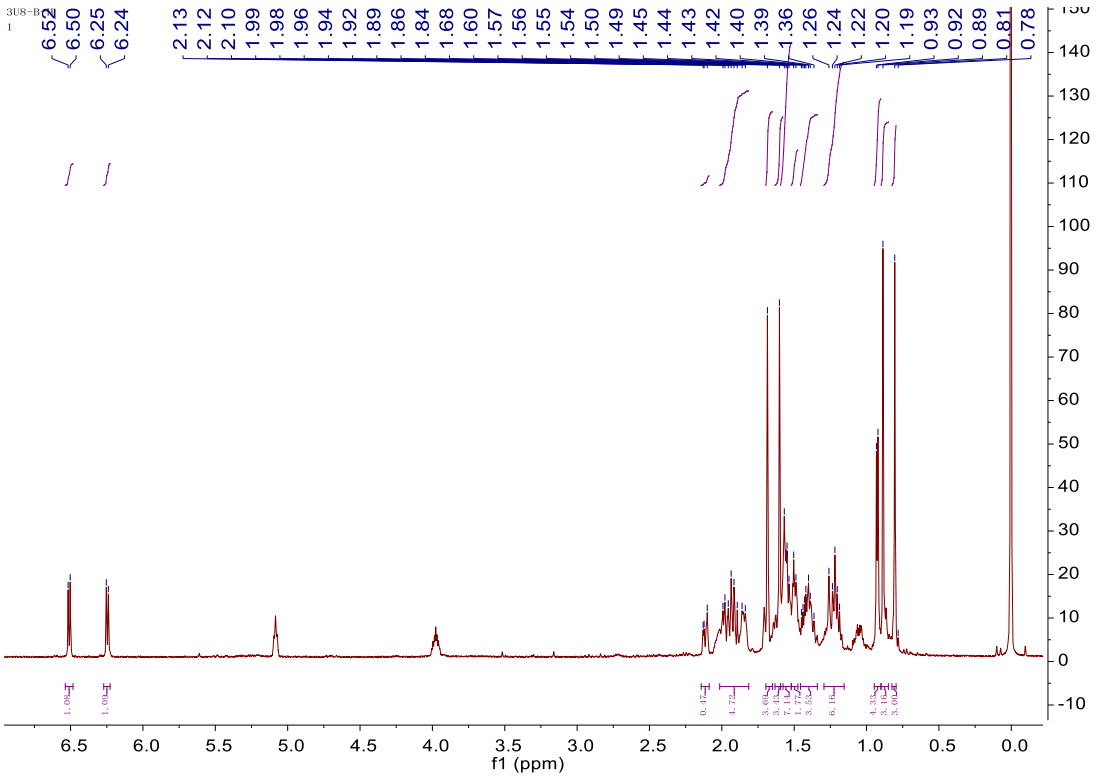
AR172 ^{13}C NMR spectrum of compound 17 in $CDCl_3$



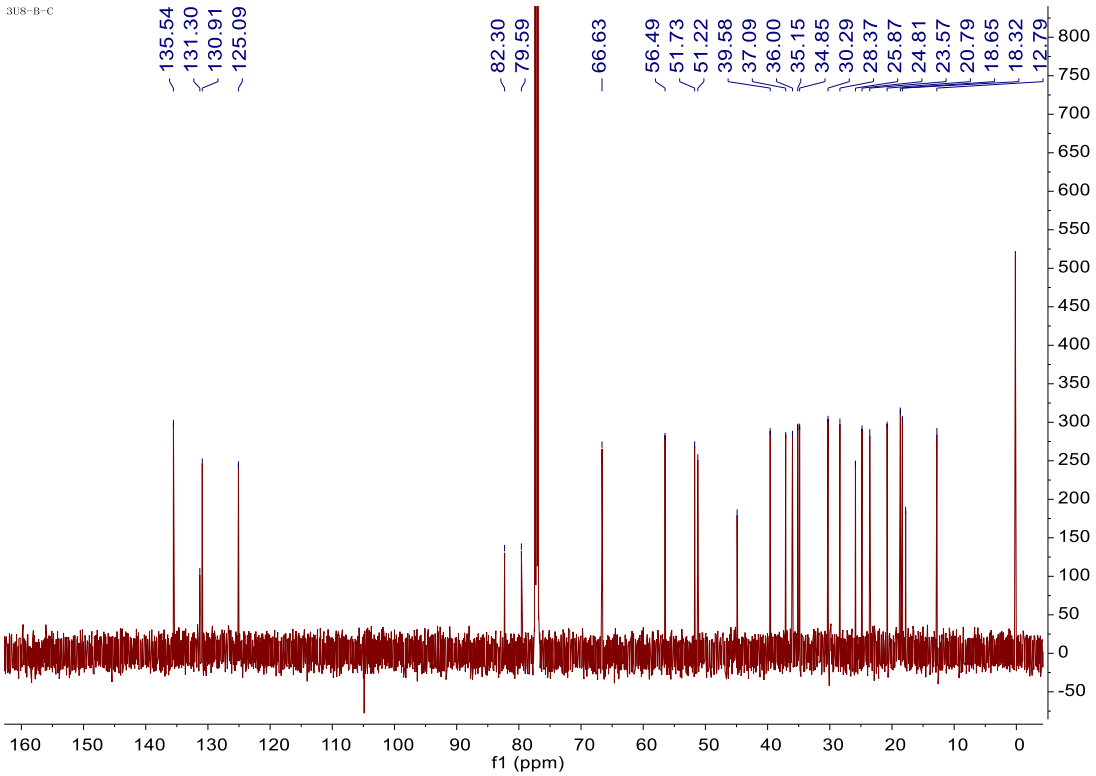
AR173 DEPT spectrum of compound 17 in $CDCl_3$



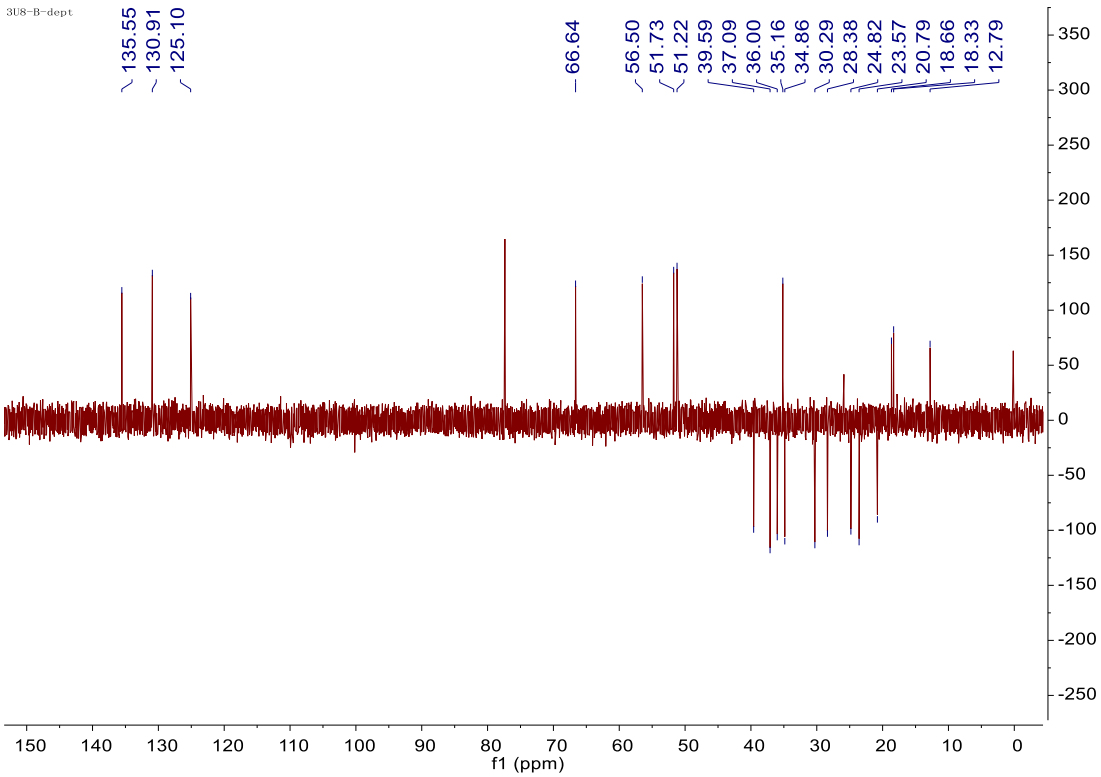
AR181 ^1H NMR spectrum of compound 18 in $CDCl_3$



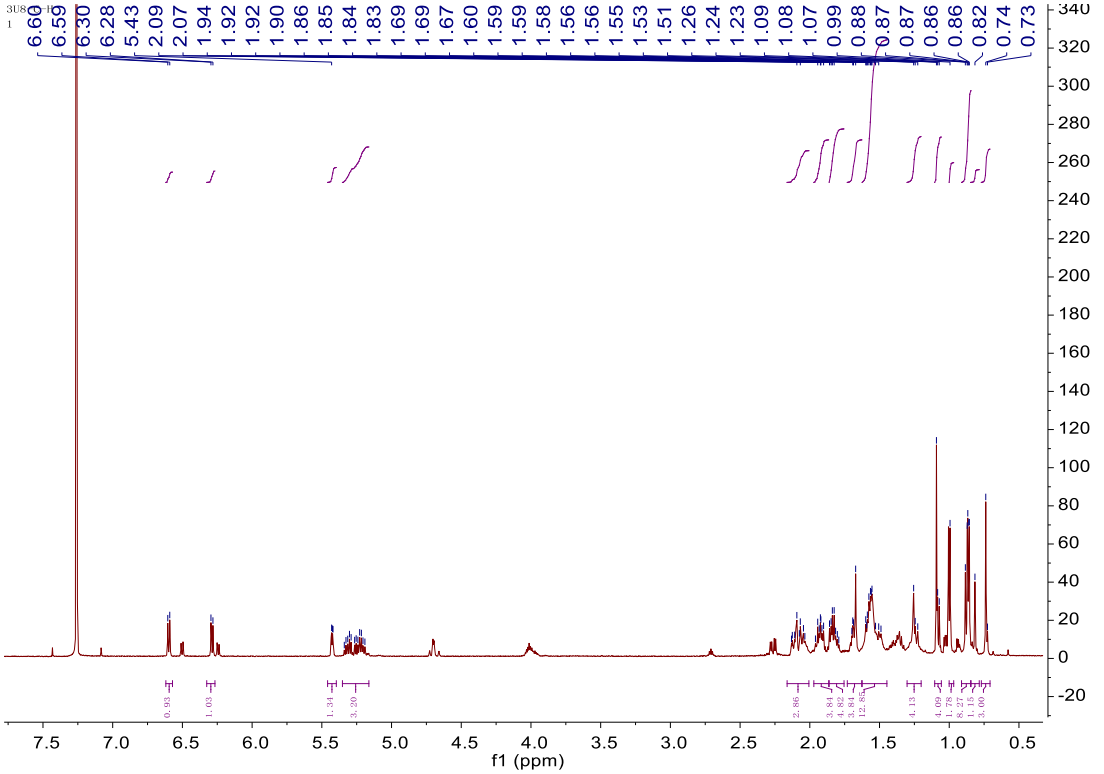
AR182 ^{13}C NMR spectrum of compound 18 in $CDCl_3$



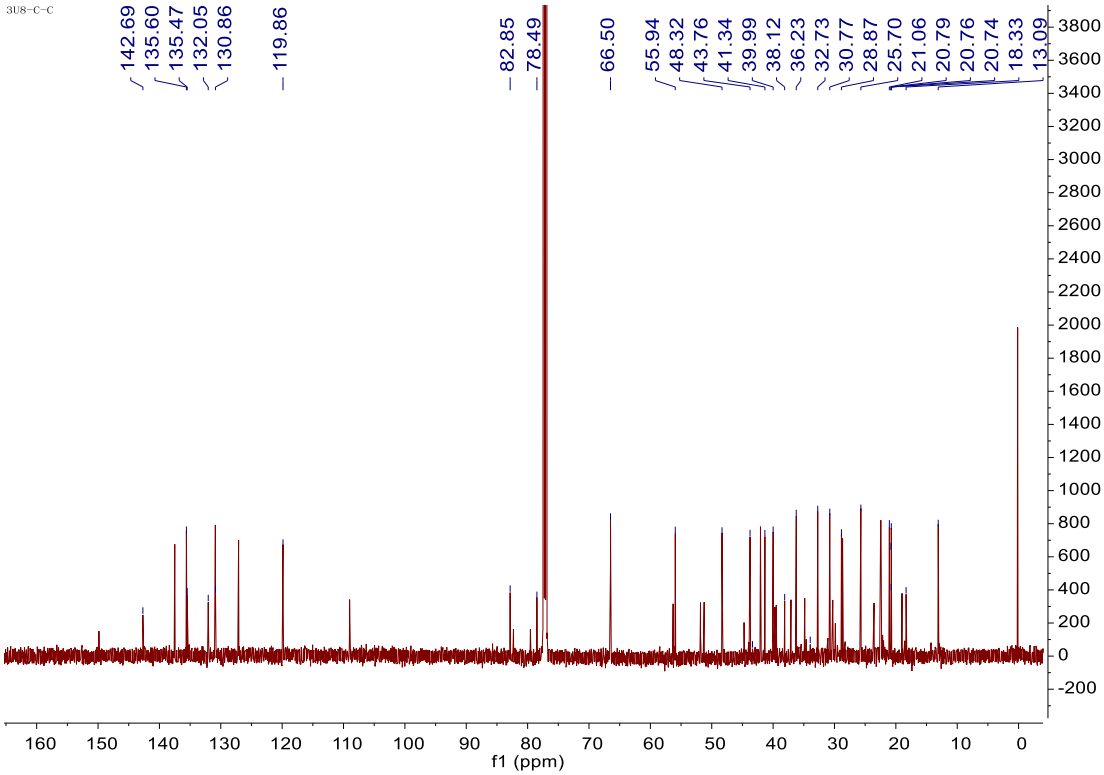
AR183 DEPT spectrum of compound 18 in $CDCl_3$



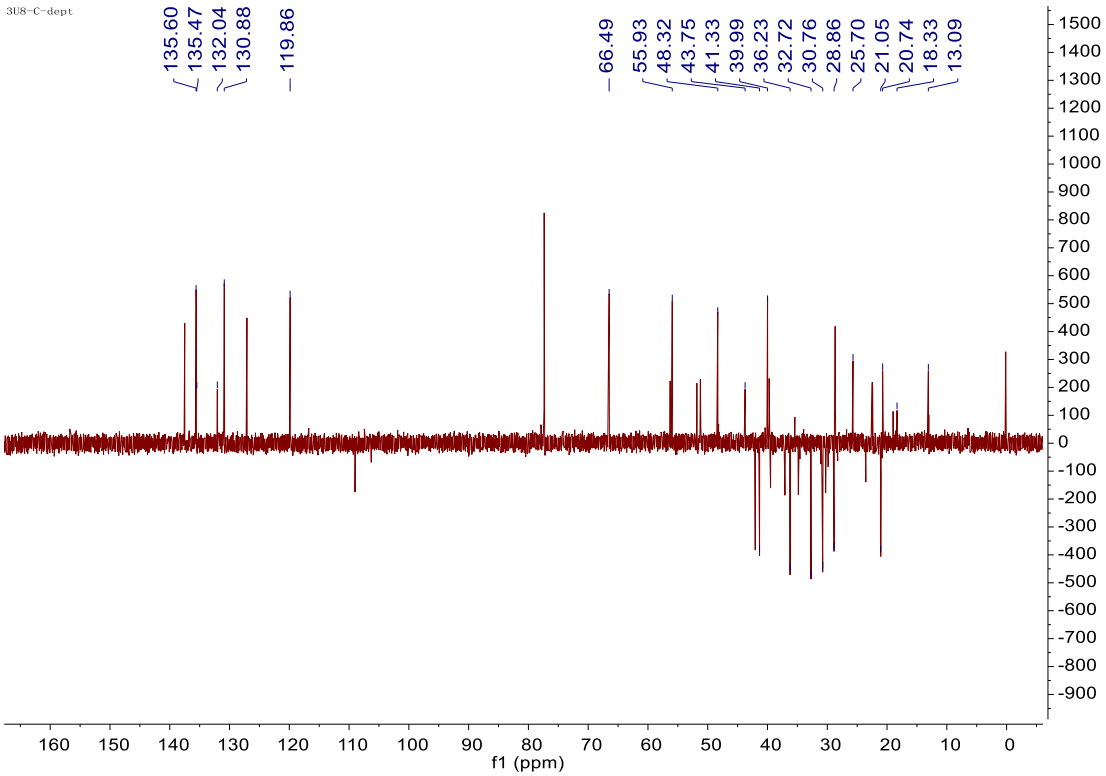
AR191 ^{1}H NMR spectrum of compound 19 in $CDCl_3$



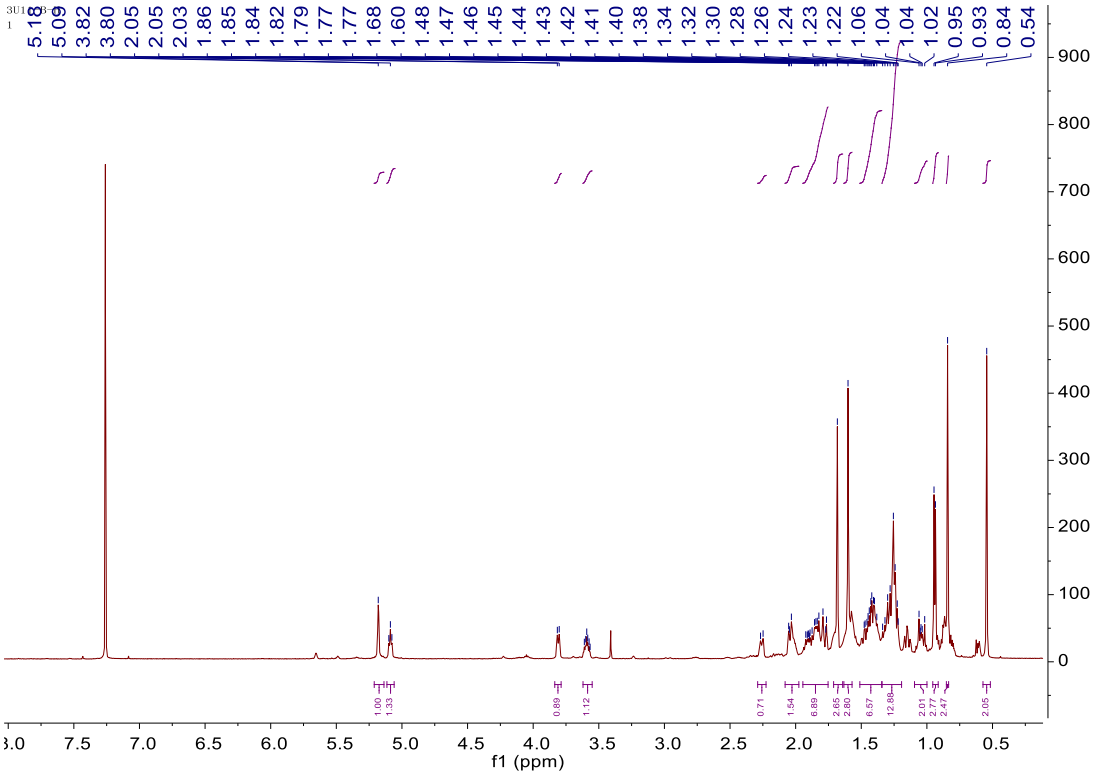
AR192 ^{13}C NMR spectrum of compound 19 in $CDCl_3$



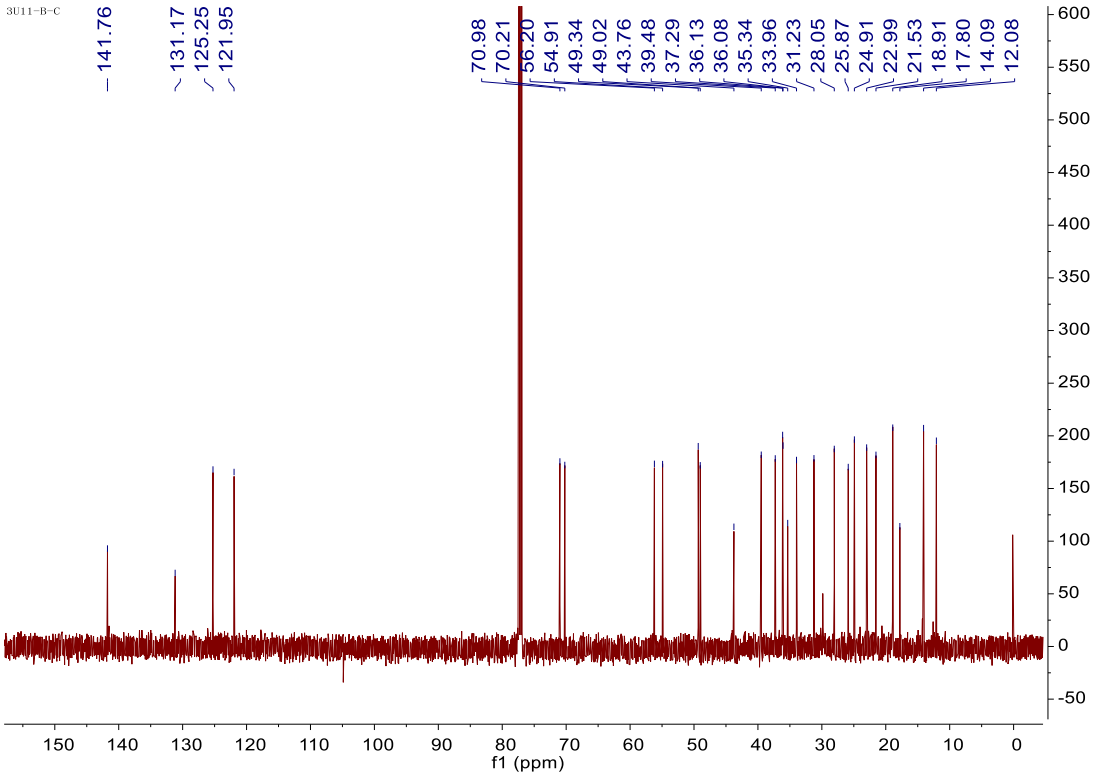
AR193 DEPT spectrum of compound 19 in $CDCl_3$



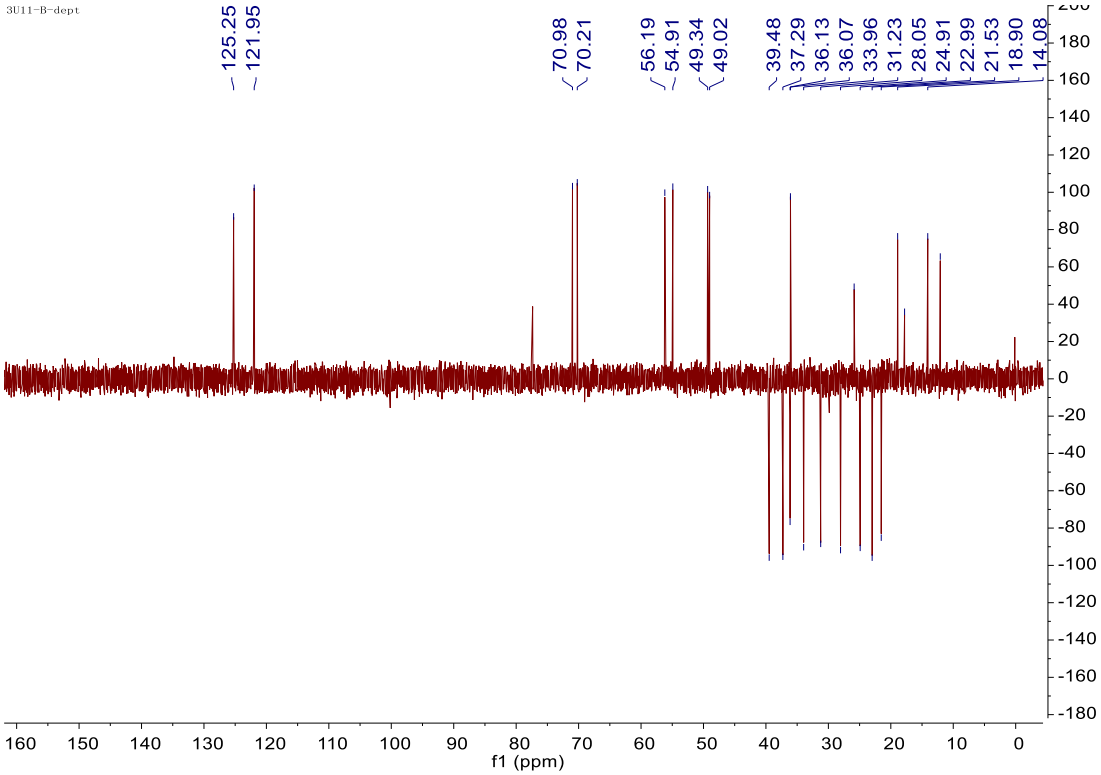
AR201 ^1H NMR spectrum of compound 20 in $CDCl_3$



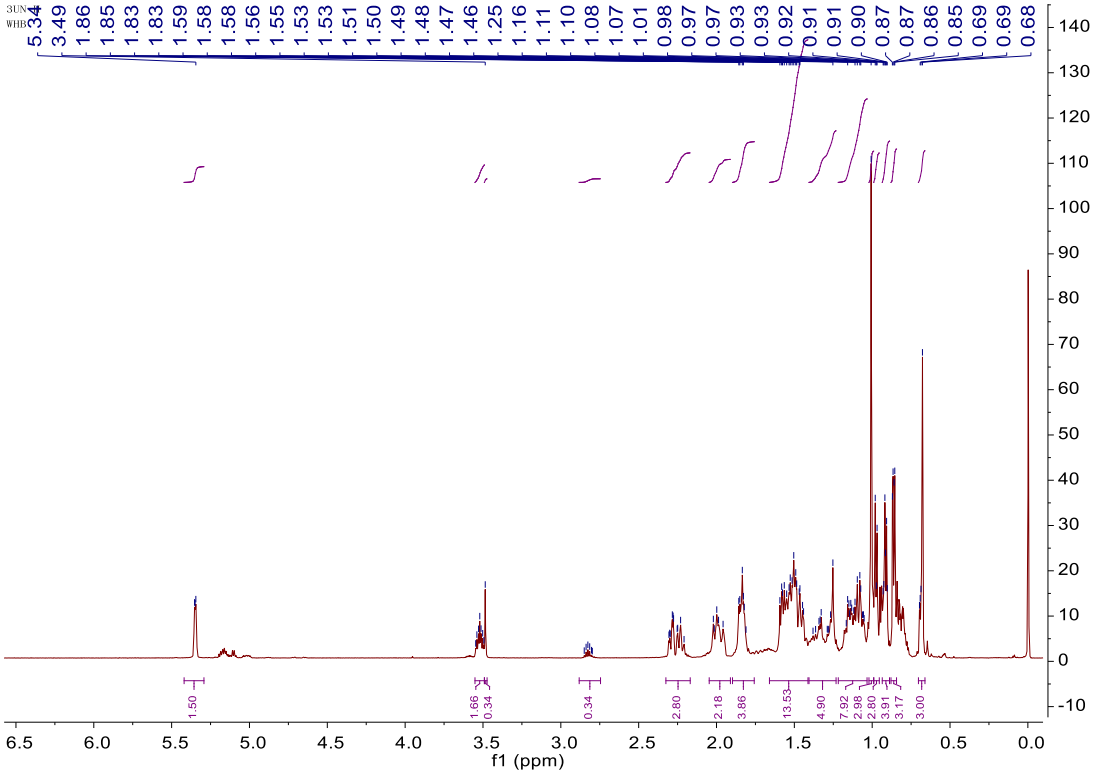
AR202 ^{13}C NMR spectrum of compound 20 in $CDCl_3$



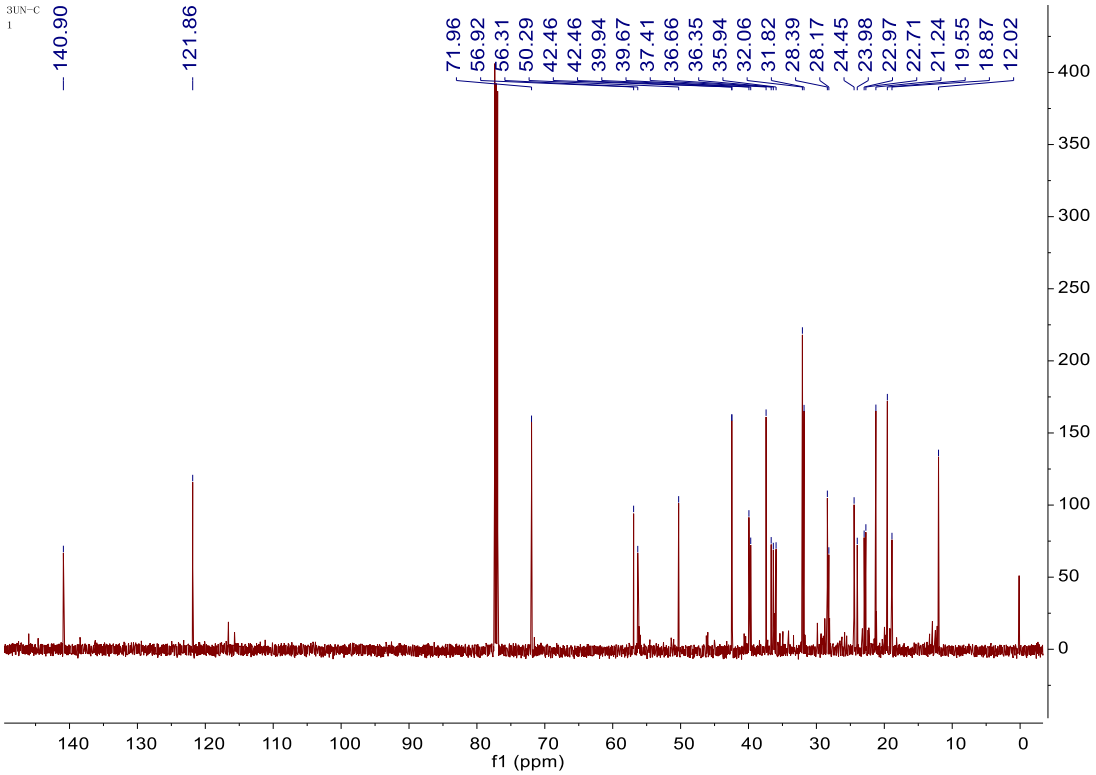
AR203 DEPT spectrum of compound 20 in $CDCl_3$



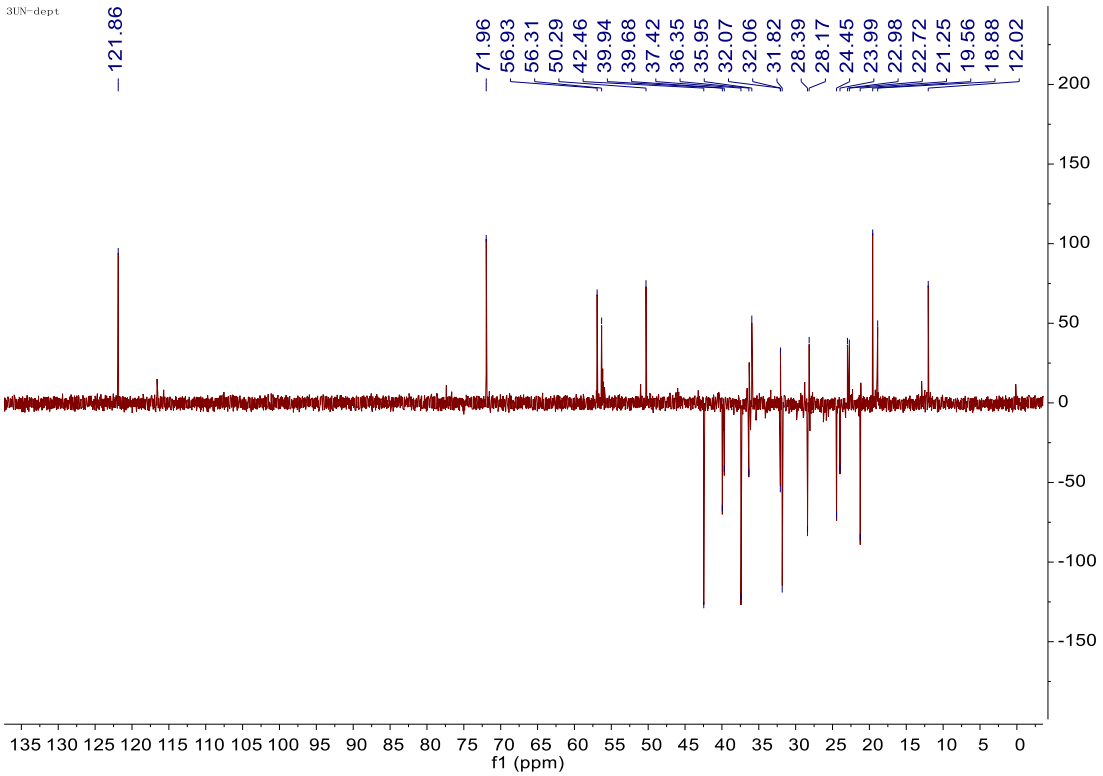
AR211 ^{1}H NMR spectrum of compound 21 in $CDCl_3$



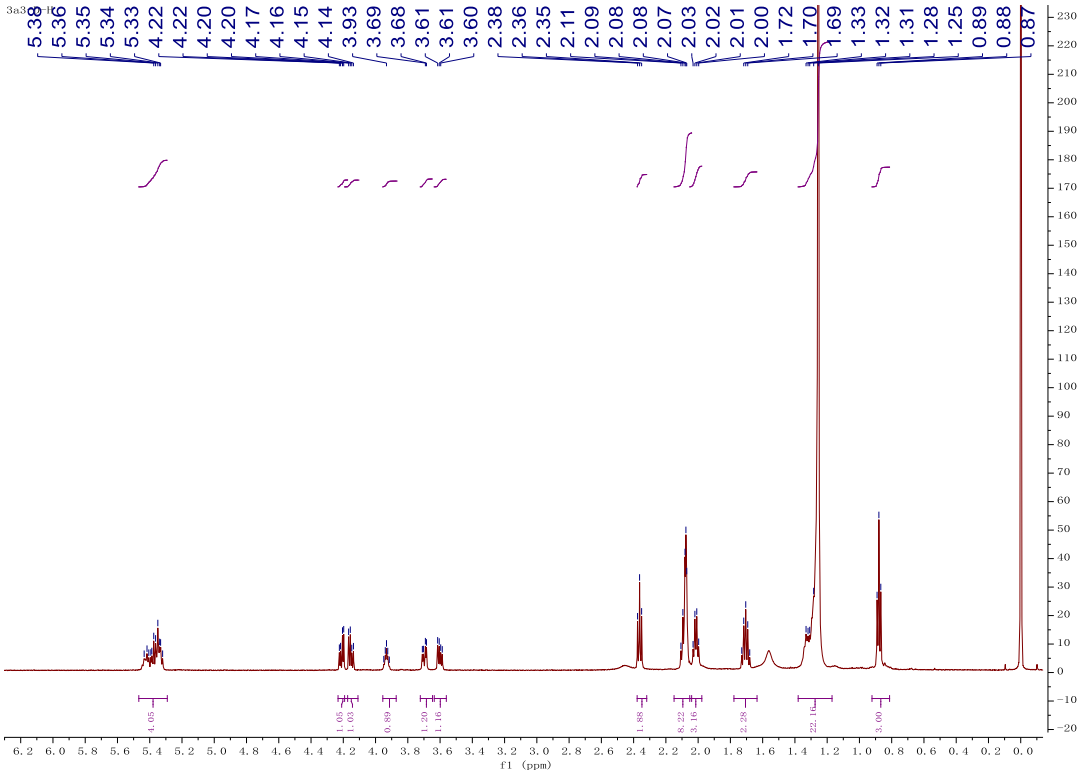
AR212 ^{13}C NMR spectrum of compound 19 in $CDCl_3$



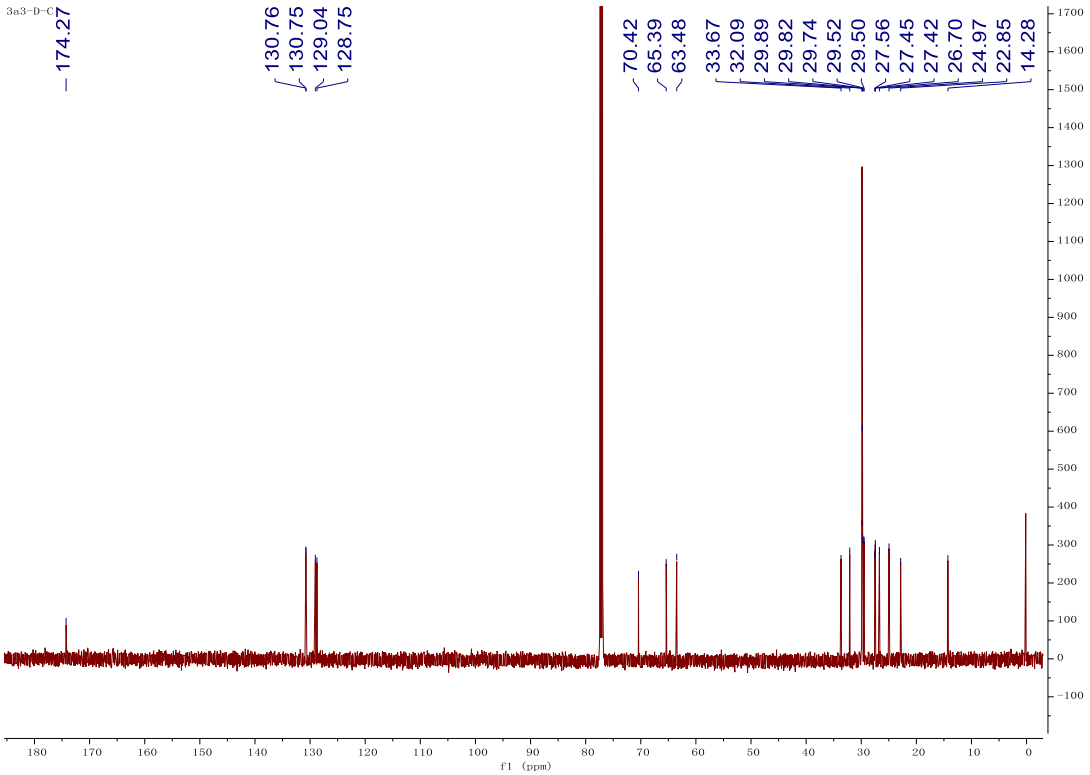
AR213 DEPT spectrum of compound 22 in $CDCl_3$



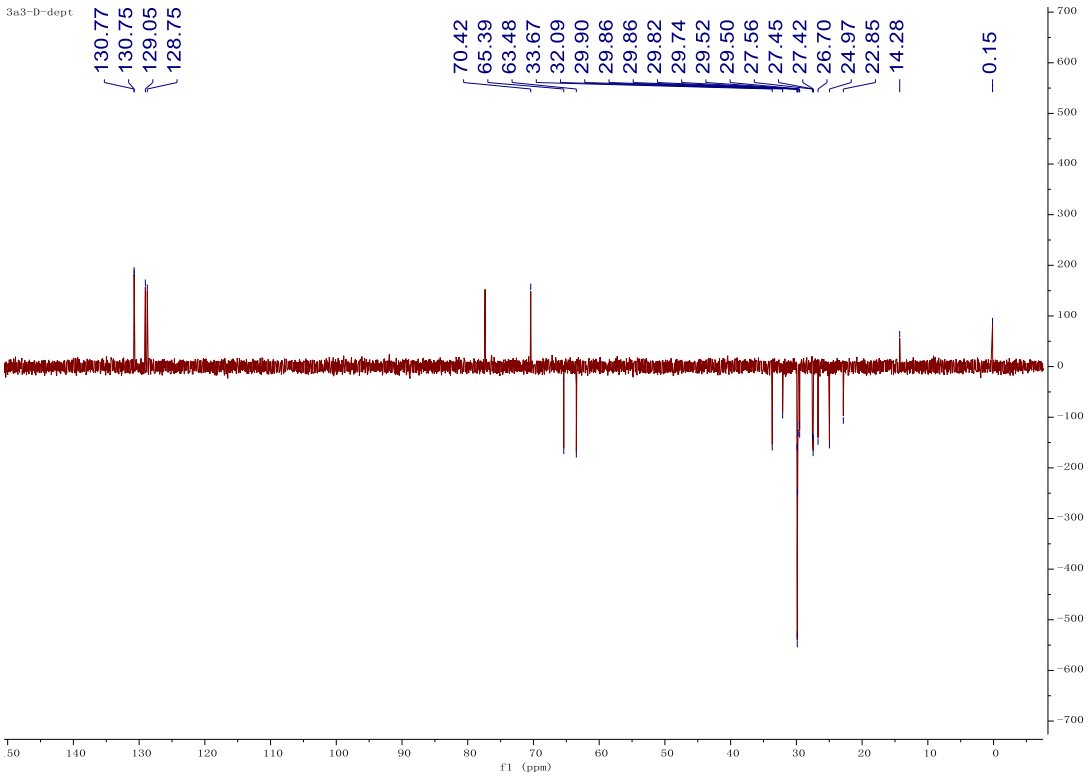
AR221 ^1H NMR spectrum of compound 22 in $CDCl_3$



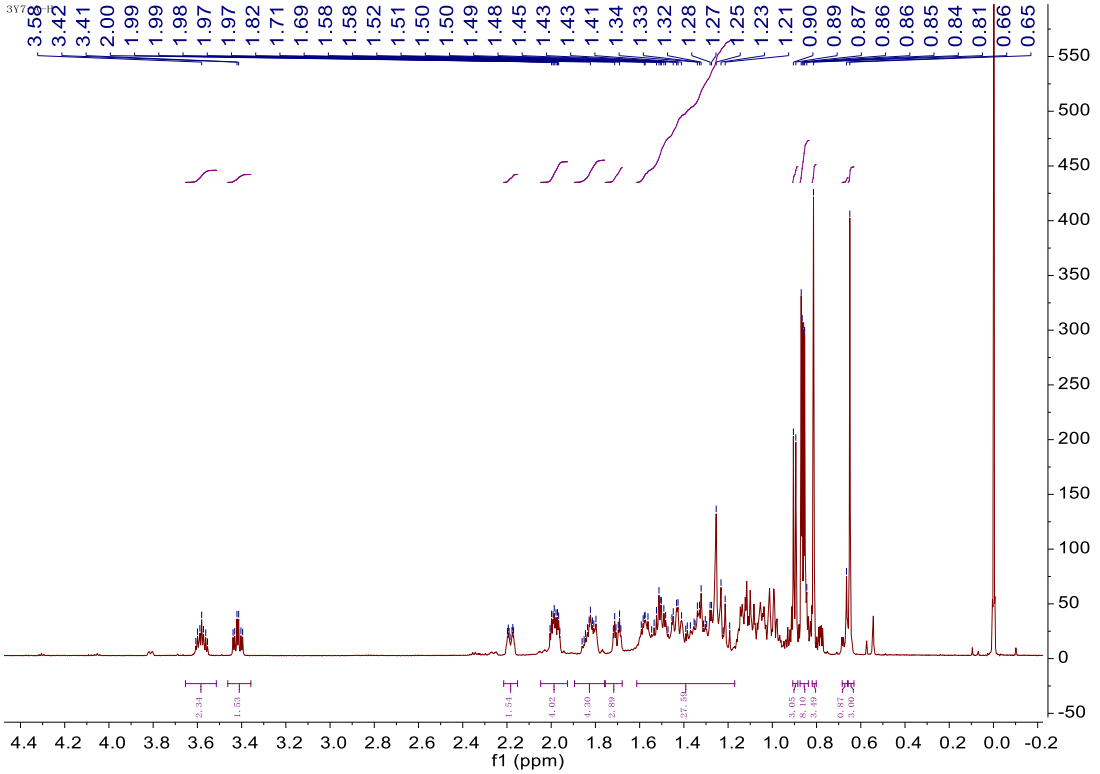
AR222 ^{13}C NMR spectrum of compound 22 in $CDCl_3$



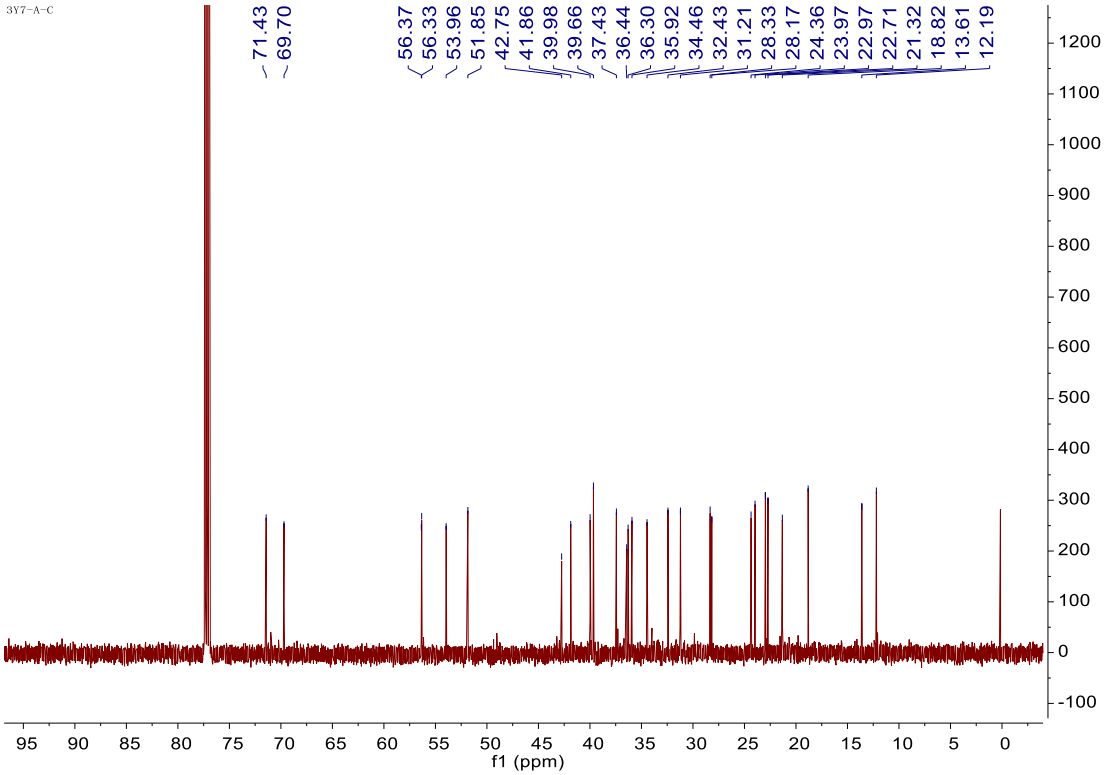
AR223 DEPT spectrum of compound 22 in $CDCl_3$



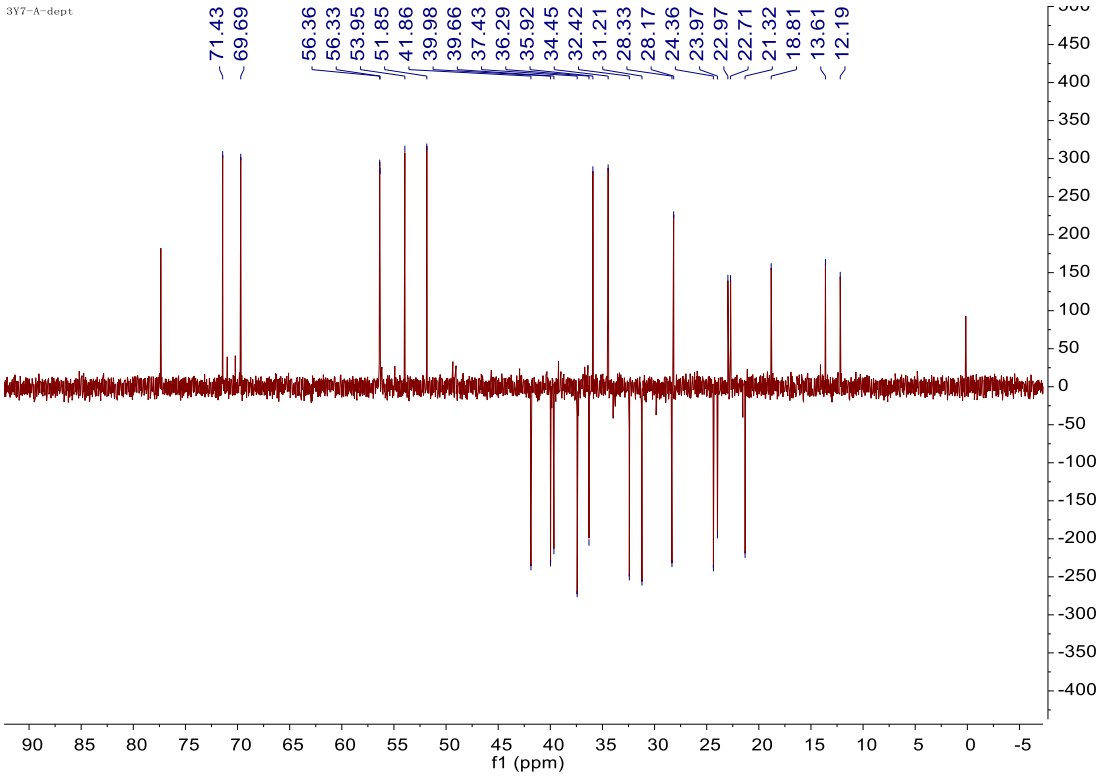
AR231 ^{1}H NMR spectrum of compound 23 in $CDCl_3$



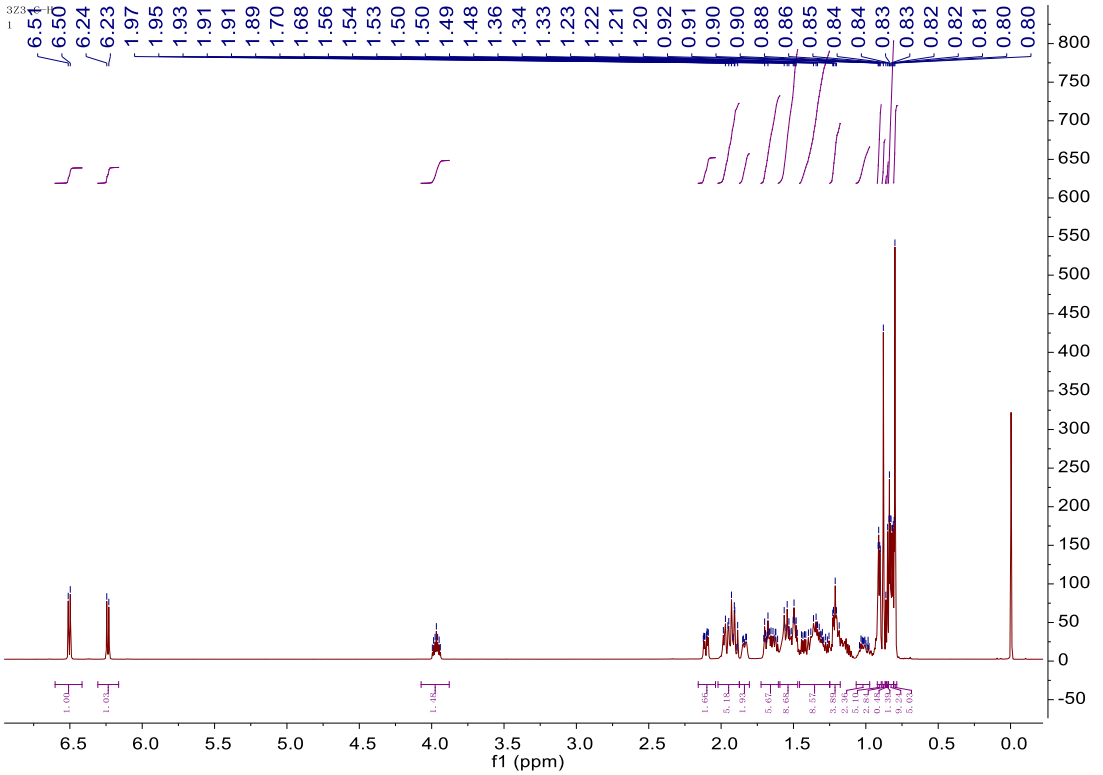
AR232 ^{13}C NMR spectrum of compound 23 in $CDCl_3$



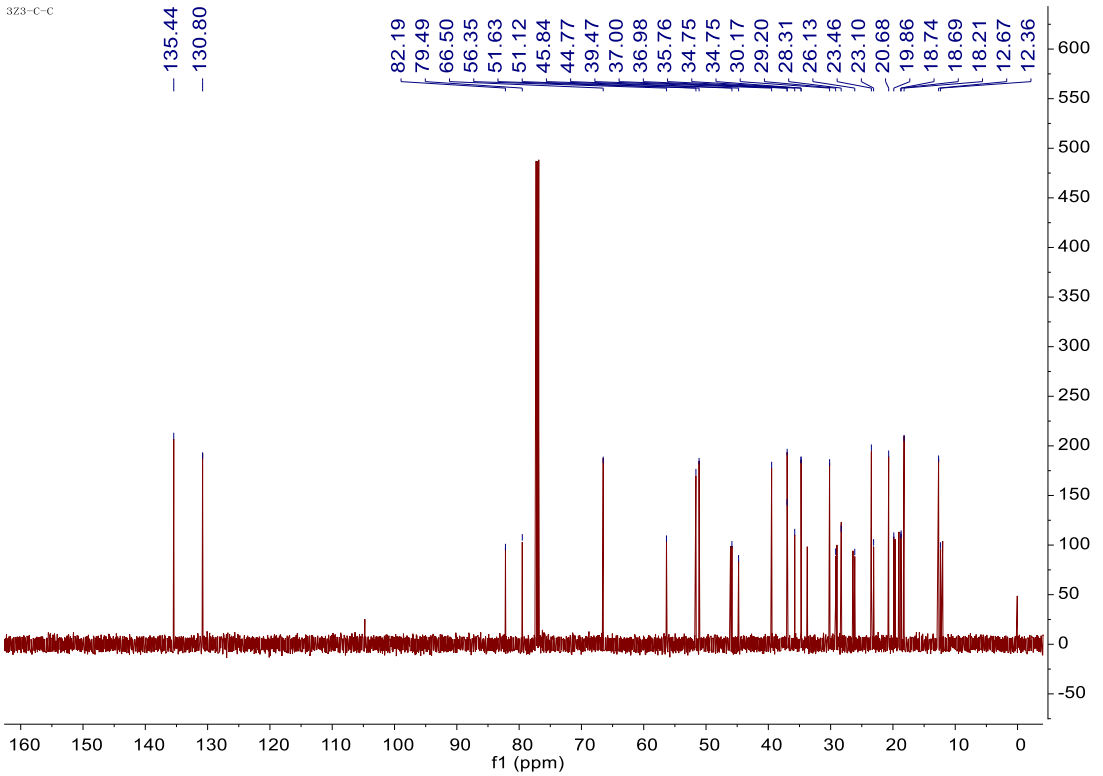
AR233 DEPT spectrum of compound 23 in $CDCl_3$



AR241 ^1H NMR spectrum of compound 24 in $CDCl_3$



AR242 ^{13}C NMR spectrum of compound 24 in $CDCl_3$



AR243 DEPT spectrum of compound 24 in $CDCl_3$