Chapter 3

Isolation, identification and bioactivity of a novel chlorophenol derivative from *Helichrysum aureonitens* cell suspension cultures

3.1 Abstract

A chlorophenol, 4-chloro-2-(hepta-1,3,5-triyne-1-yl)-phenol, was isolated as the major phenolic compound from the *Helichrysum aureonitens* cell suspension culturess. This triyne has been proposed to be an intermediate in the acetylene biosynthetic pathway in *Helichrysum* spp., but only the methyl ether form has previously been isolated from the roots of *H. coriaceum*. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the ethanol extracts of cell suspension cultures of *H. aureonitens* against *Mycobacterium tuberculosis* H37Rv were found to be 1.0 mg/ml and 2.0 mg/ml respectively and the triyne was not active at 200 µg/ml. The ethanol extract of the cell suspension cultures and the triyne were evaluated for their cytotoxicity against monkey kidney (Vero cells) and human prostate epithelial carcinoma (DU145) cell lines. The inhibitory concentrations (IC_{50}) of the triyne and the crude extract were found to be 1.51 and 12.11 µg/ml against Vero cells respectively. The crude extract and the triyne showed similar activity in the prostate cancer cell lines by exhibiting IC_{50} values of 3.52 and 2.14 µg/ml respectively. The triyne warrants further investigation for its potential as an anticancer drug.

3.2 Introduction

According to the medicinal properties of *Helichrysum aureonitens* described in previous chapters, it can be considered as an important medicinal plant, which could benefit human health. One of the most efficient ways to produce secondary plant products is by a cell suspension culture system. Some plant cell suspension cultures produce the same secondary metabolites as the parent plants, while others produce different profiles in culture systems (Khafagi et al., 2003). This method offers an alternative opportunity to produce secondary metabolites by changing some culturing factors, plant growth regulators and elicitors or nutritional factors *in vitro*. This process can up-regulate or down-regulate the expression of genes involved in different biosynthetic pathways and consequently produce higher quantities of metabolites (Rao and Ravishankar, 2002). Research has shown that the cell suspension culture system can induce new compounds, not previously observed in the intact plants (Monache et al., 1994). A good example would be *Sophora flavescens*. Application of cork tissue and/or methyl jasmonate to cell suspension cultures of *S. flavescens*, not only increased the amount of sophoraflavanone G as a major flavonoid but also produced two precursors of sophoraflavanone G. Both of these precursors could not be detected either in cultured cells or in the original plants (Zhaoa et al., 2003). Elicitation of cell suspension cultures of *H. kraussii* also induced the production of two compounds which were not present in the control cultures (Prinsloo and Meyer, 2006). There are several successful cell suspension culture experiments for producing higher amount of secondary metabolites than in the intact plants. Scragg et al. (1990) reported that quinoline alkaloids were produced in significant quantities from globular cell suspension cultures of *Cinchona ledgeriana*. Significant amounts of

sanguinarine were also produced in cell suspension cultures of *Papaver somniferum* using bioreactors (Park et al., 1990).

Drapeau et al. (1987) estimated that a 40-fold increase in the ajmalicine productivity of *Catharanthus roseus* would be required to justify the production of this compound by cell culture methods. In contrast the cells of ginseng (*Panax ginseng*) callus culture did not produce polyacetylene, an anticancer compound, although it was present in the intact ginseng plants (Yang et al., 1999).

When analysing results in the previous chapter it was found that galangin was not detected in the *H. aureonitens* cell suspension cultures. However, the production of two other compounds was induced to much higher concentrations when compared to the intact plant samples.

This part of the study will focus on the isolation, identification and the structural elucidation of the compound found in the ethanol extract of the *H. aureonitens* callus. The observations on the medicinal properties of the compound of the callus cultures are also reported.

3.3 Materials and methods

3.3.1 Induction of plant callus and the establishment of cell suspension cultures

The cell suspension cultures *H. aureonitens* were induced from fully expanded leaves, which were harvested from *H. aureonitens* grown in the greenhouse as described in chapter 2. Subcultures were created at 20-day intervals by transferring 200 mg fresh cells into a 100-ml Erlenmeyer flask containing 20 ml fresh medium and incubated on a shaker at 25 °C in the dark.

3.3.2 Extraction

Fresh leaves from greenhouse grown plants and cells from cell suspension cultures of *H. aureonitens* were extracted with pure ethanol.

3.3.3 Thin layer chromatography (TLC)

Thin layer chromatography (TLC) was used as a preliminary step for the detection of compounds in all extracts and fractions (eg. galangin). The samples were spotted on TLC plates, developed in a hexane: dichloromethane: methanol system (10: 10: 0.5) followed by spraying with 0.34 % vanillin in 3.5 % sulphuric acid in methanol.

Two dimensional TLC analysis was carried out to determine the degradation of compounds. Second development was done immediately after the first one finished in a hexane: ethyl acetate system (7: 1). The main and decomposed spots were visualized under UV light. Control TLC plates were developed with a similar procedure, but without UV irradiation. To visualize the spots on control TLC plates, *p*-anisaldehyde was used.

3.3.4 Isolation and purification and identification of the compound from the ethanol extract of the cell suspension cultures

In a preliminary fractionation of compounds in the crude ethanolic extracts of *H. aureonitens* cell suspension cultures, the samples were applied to a Sephadex column (LH-20, Sigma-Alderich, South Africa) and eluted with 95 % ethanol. The eluted fractions were developed on TLC plates and the fractions that contained major compounds were collected and further purified using prep-TLC under dark conditions. The solvent system for prep-TLC developing was hexane: ethyl, acetate (7:1).

The fractions containing the major compounds were pooled together and column chromatograghy was again performed on a Sephadex LH-20 column using 95 % ethanol as eluent. The partially pure compound obtained from the Sephadex column was finally purified using prep-TLC in a hexane: dichloromathane: methanol system (10: 10: 0.5).

The chemical structure of the isolated compound was identified by applying different spectroscopic means including NMR (1D and 2D) IR, MS and UV.

3.3.5 Identification of the isolated compound from the cell suspension cultures

GC-MS analysis was carried out as described in chapter 2. Briefly a mass spectrometer (JMS-AM SUN200, JEOL) connected to a gas chromatograph (6890A, Agilent Tecnologies) with the following parameters, EI (70 eV), source temperature 250 °C, HP-5 column (30 m x 0.25 mm, 0.32 μm film thickness, J&W Scientific), injection temperature 250 °C, column temperature program: 80 °C for 1 min, then raised to 300 °C at a rate of 10 °C min^{-1}, and held on this temperature for 10 min; interface temperature 280 °C, carrier gas He, flow rate 1.2 mL min^{-1}, splitless injection.

3.3.6 Bioassays

3.3.6.1 Antituberculosis activity

The radiometric respiratory technique using the BACTEC system (Becton Dickinson Diagnostic Instrument, Spars, md) was used for testing susceptibility of *Mycobacterium tuberculosis* H37Rv (ATCC 27264) using the method described by Heifets and Good (1994). Solutions of the cell suspension culture and compound **1** were prepared by maceration of a requisite amount of the sample in a known volume of dimethyl sulphoxide (DMSO) to obtain a concentration of 5.0 mg/ml for both the crude extract and compound **1**. The solutions were stored at 4 ˚C until used. Subsequent dilutions were done in DMSO and added to 4.0 ml of BACTEC 12B (7H12 medium) broth to achieve the desired final concentrations of 2.0, 1.0, and 0.5 mg/ml for the crude extract and 200, 100, 50 µg/ml for compound **1**, with PANTA (Becton Diskinson & Company), an antimicrobial supplement. Control experiments showed that the final amount of DMSO (1 %) in the media had no effect on the growth of *M. tuberculosis*.

The radiometric respiratory techniques using the BACTEC 460 system (Becton Dickinson Diagnostic Instrument, Sparks, md) was used for testing susceptibility against *M. tuberculosis* as previously described (Mativandlela et al., 2006). Isoniazid (INH) (Sigma-Aldrich, South Africa) at a final concentration of 0.2 µg/ml served as the drug-control. The MIC was defined as the lowest concentration of the compound that inhibited more than 99 % of the bacterial population. The bactericidal effect (minimum bactericidal concentration, MBC) of the extract was assessed by plating the bacterial suspensions from the BACTEC vials, which exhibited a MIC effect, on 7H11 agar medium at the end of the experiment. The MBC was defined as the minimal bactericidal concentration, which effectively reduced by at least 99 % the viable

counts in the extract or compound containing samples as compared with those in the control vials (extract and compound free vials). The experiment was repeated three times.

3.3.6.2 Cytotoxicity assay

Microtitre well plates with Vero and human prostate epithelial carcinoma (DU145) cells were used for toxicity analysis of the ethanolic crude extract and the triyne according to Zheng et al. (2001). The positive toxicity drug, Zelaraleonone was tested at the final concentrations of 7.52 - 0.26 µg/ml. Cytotoxicity was measured by the XTT (sodium 3'-[1-(phenyl amino-carbonyl)-3,4-tetrazolium]-bis-[4-methoxy-6-nitro] benzene sulfonic acid hydrate) method using the cell proliferation kit II (Roche Diagnostics GmbH). Vero and DU145 cells (100 µl) were seeded at 1 x 10^5 into microtitre plates and incubated for 24 h. A dilution series was made of the *H. aureonitens* cell suspension crude extract (400.0 to 3.125 µg/ml) and the triyne (200.0 to 1.563 µg/ml), and was added to the microtitre plates and incubated for 72 hrs. Samples were used in triplicates. The absorbance of the colour complex was quantified at 490 nm using an ELISA plate reader with a reference wavelength set at 690 nm. The IC_{50} value was defined as the concentration of the compounds at which absorbance was reduced by 50 %. The 'GraphPad Prism 4', statistical program was used to analyze the 50 % inhibitory concentration (IC_{50}) values (Motulsky, 2003).

3.4 Results

3.4.1 Isolation and purification of compound 1

Figure 3.1 shows the TLC chromatogram of the fractios of *H. aureonitens* cell suspension crude extracts that were eluted from the Sephadex column containing two major compounds. In most of the fractions both compounds **1** and **2** eluted together. But only compound **1** could be isolated and purified after column chromatography.

3.4.2 Identification of compound 1

Compound **1** was isolated as a semi-solid from the non-polar part of the ethanol extract of *H. aureonitens* cell suspension cultures. HRESI (negative mode) gave 213.0181 $(M-1)^+$ corresponding to the molecular formula $C_{13}H_7OCl$. ^1H NMR showed four signals at 7.33 (b s), 7.23 (d, 7.8 Hz), and 6.86 (d, 7.8 Hz), in addition to the methyl singlet signal at 2.05. The ^{13}C NMR showed 13 carbons, six of them were aromatic 157.4, 132.2, 131.6, 125.2, 116.6, 109.2, six carbons of acetylenic bonds at 82.2, 80.1, 70.1, 68.1, 64.6, 57.7 and a signal of methyl carbon at 4.7. HMBC cross-peak showed a correlation of H-3/ C-1, C-2, C-4, C-5, C-7, H-4/ C-3, C-2, C-5, C-6 and H-6/ C-1 C-2, C-5, C-4. The above data indicated the compound to be 4-chloro-2-(hepta-1,3,5-triyne-1-yl)-phenol (Fig. 3.2 and 3.3). The other data which included 2D NMR, HMQC, HMBC and COSY experiments also supported the structure of compound **1** (Fig. 3.4 to 3.8 and Table 3.1).

3.4.3 GC-MS analysis of detection of the triyne in the cells and leaves sample

Figure 3.9 shows the comparison of the GC-MS analyses of the triyne content of the cells of suspension cultures and leaves of *H. aureonitens*. It indicates that the triyne is also present in the leaves sample, but at very low concentrations.

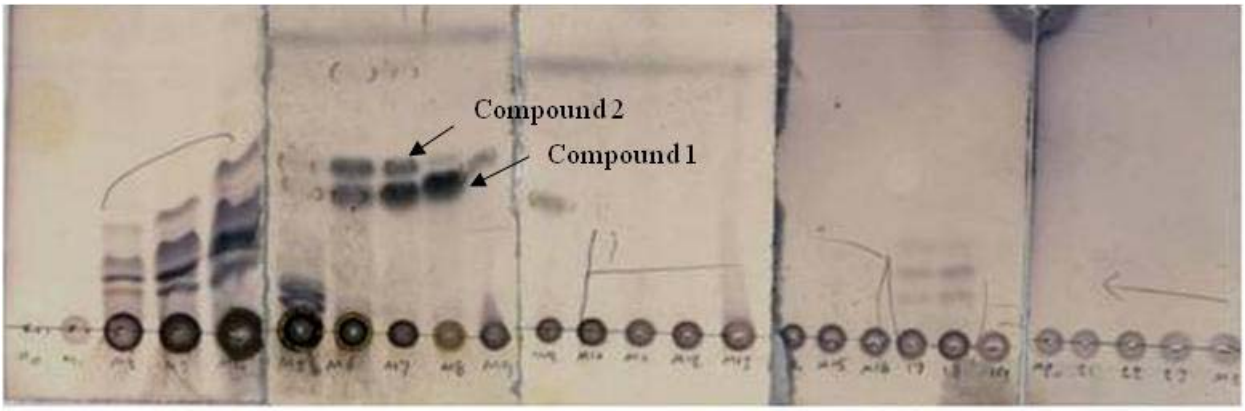

Fig. 3. 1. Results of developed collected fractions from the Sephadex column on TLC plates. Arrows show the presence of two compounds in some of the fractions of the cell suspension culture extracts which were not observed in the leaf samples of *H. aureonitens*.

Table 3. 1: NMR data of the isolated compound ($CDCl_3$, 300 MHz/75 MHz).

C	δ_C	δ_H	HMBC
	1 157.45		
	2 109.22		
	3 132.20	7.330 (d, 2.4)	C-1, -4, -5, -7
	4 125.24		
	5 131.63	7.226 (dd, 5.7, 2.4)	C-1, -2, -3, -4, -6
	6 116.59	6.867 (d, 5.7)	C-1, -2, -4, -5, -7
	7 68.09		
	8 57.76		
	9 64.58		
	10	70.08	
	11	80.09	
	12	82.05	
	13	4.72 2.057 (s)	C-8, -9, -10, -11, -12
-OH		5.739 (s)	

Coupling constants are expressed in Hz.

Carbon assignments from C-8 to 12 were not complete.

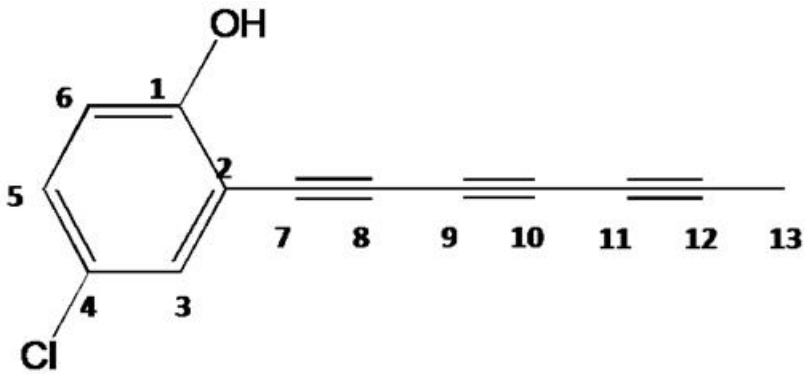

Fig. 3. 2. Chemical structure of the major compound based on the results of NMR (Carbon number) (4-chloro-2-(hepta-1,3,5-triyne-1-yl)-phenol).

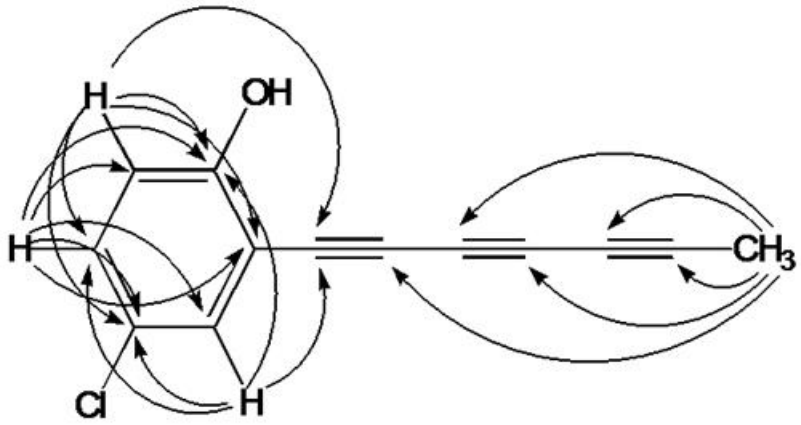

Fig. 3. 3. Chemical structure of the major compound based on the result of NMR (HMBC correlation).

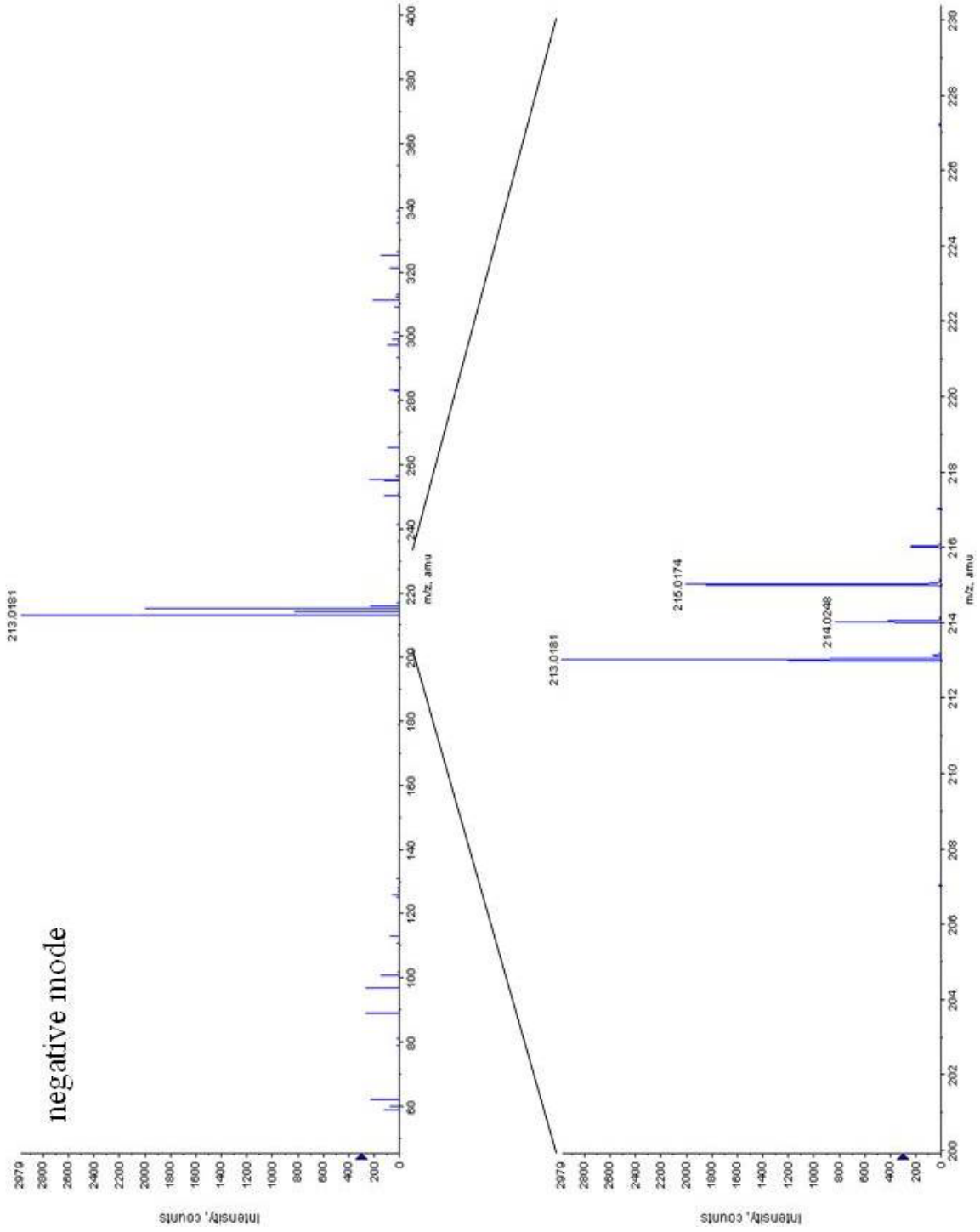

Fig. 3. 4. MS spectrum of the triyne using ESI.

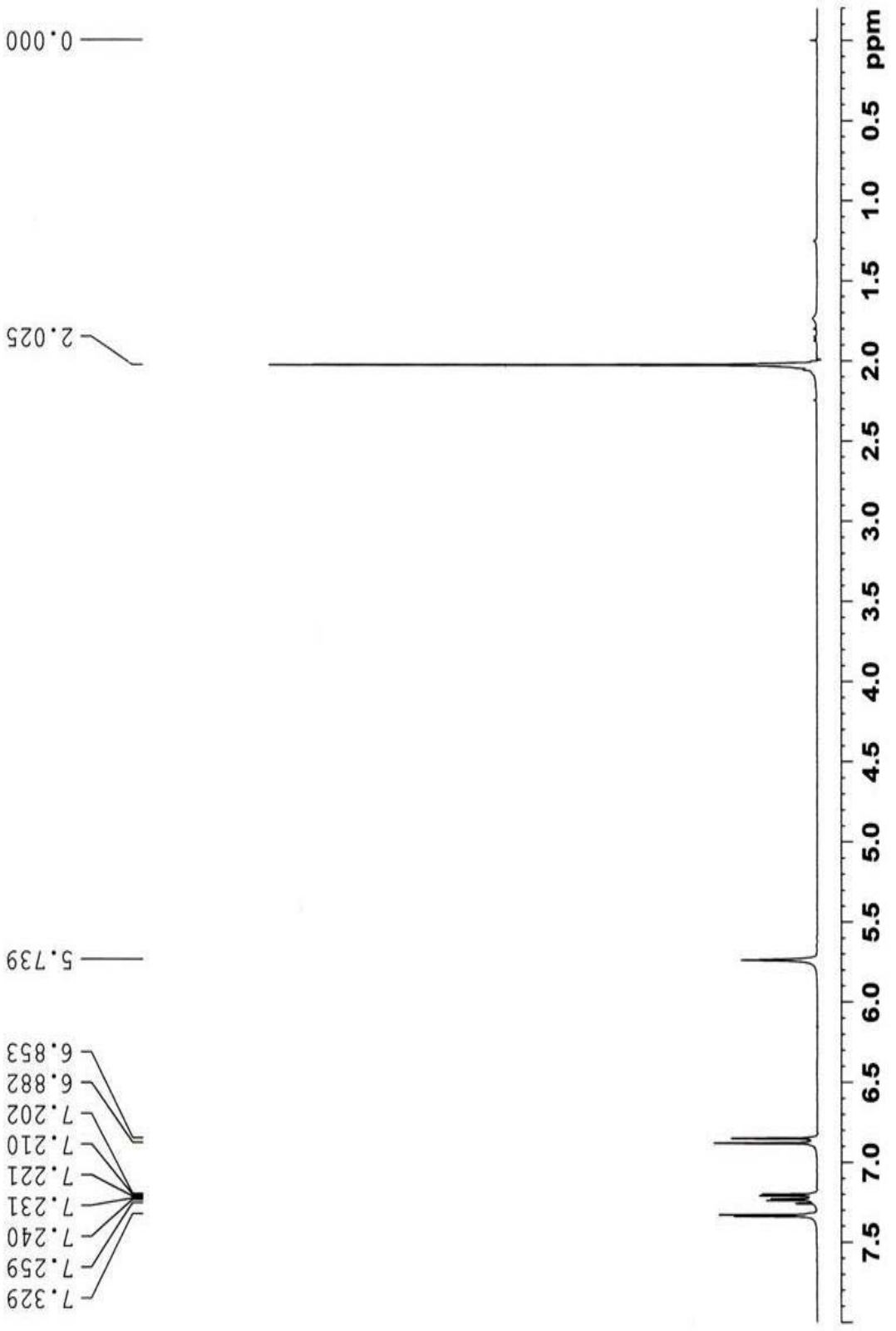

Fig. 3. 5. ^{1}H-NMR of the triyne

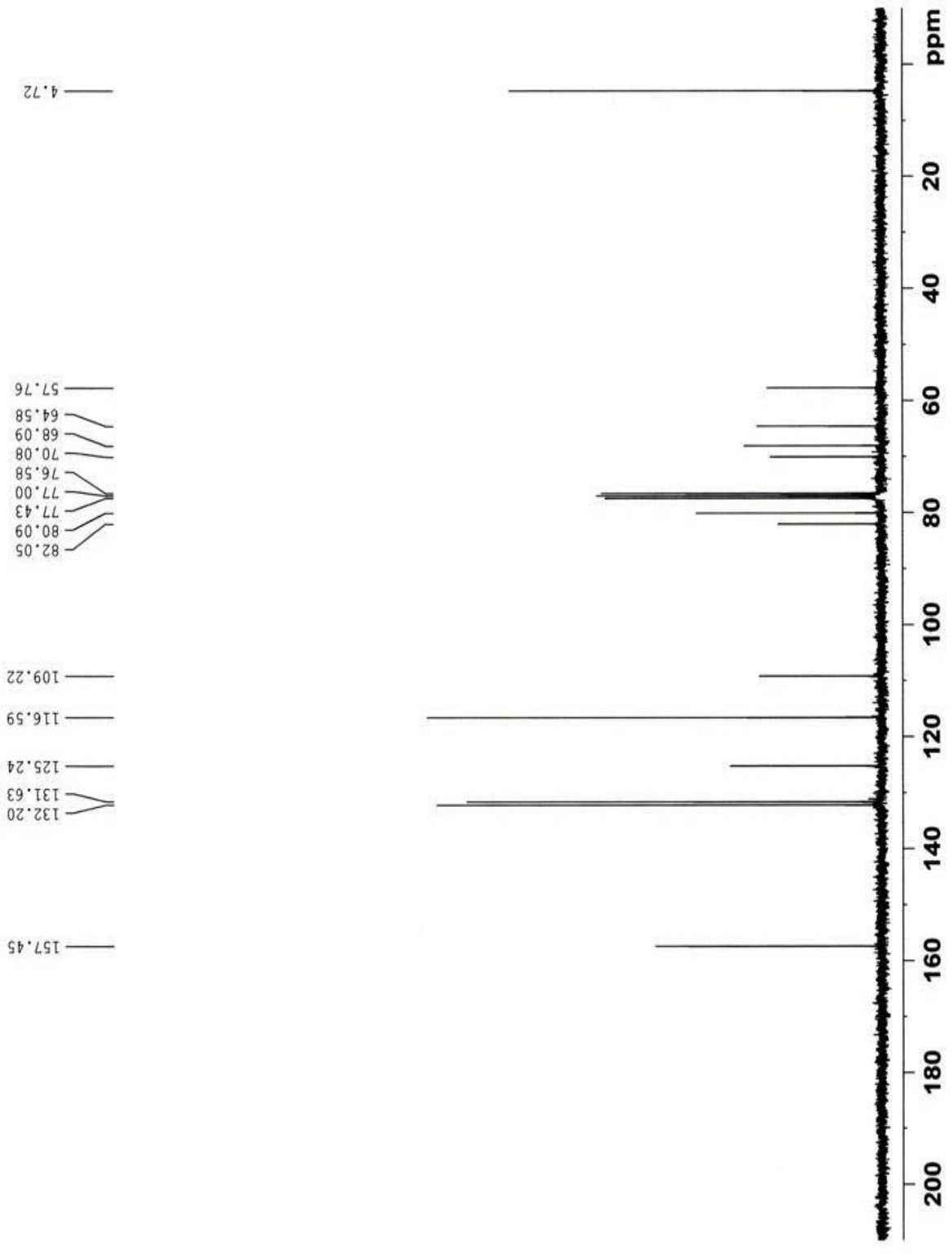

Fig. 3. 6. ^{13}C-NMR of the triyne.

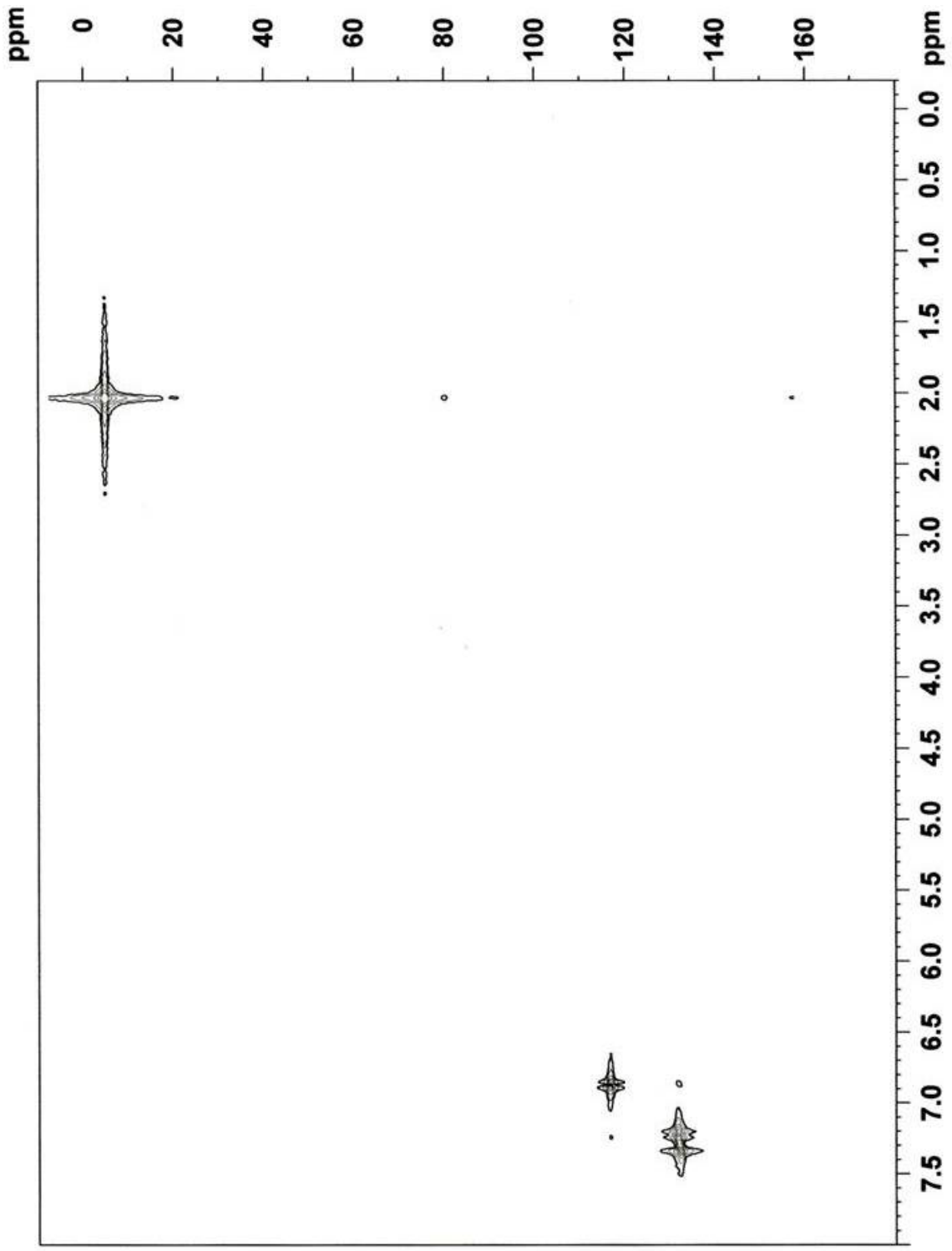

Fig. 3. 7. HMQC of the triyne.

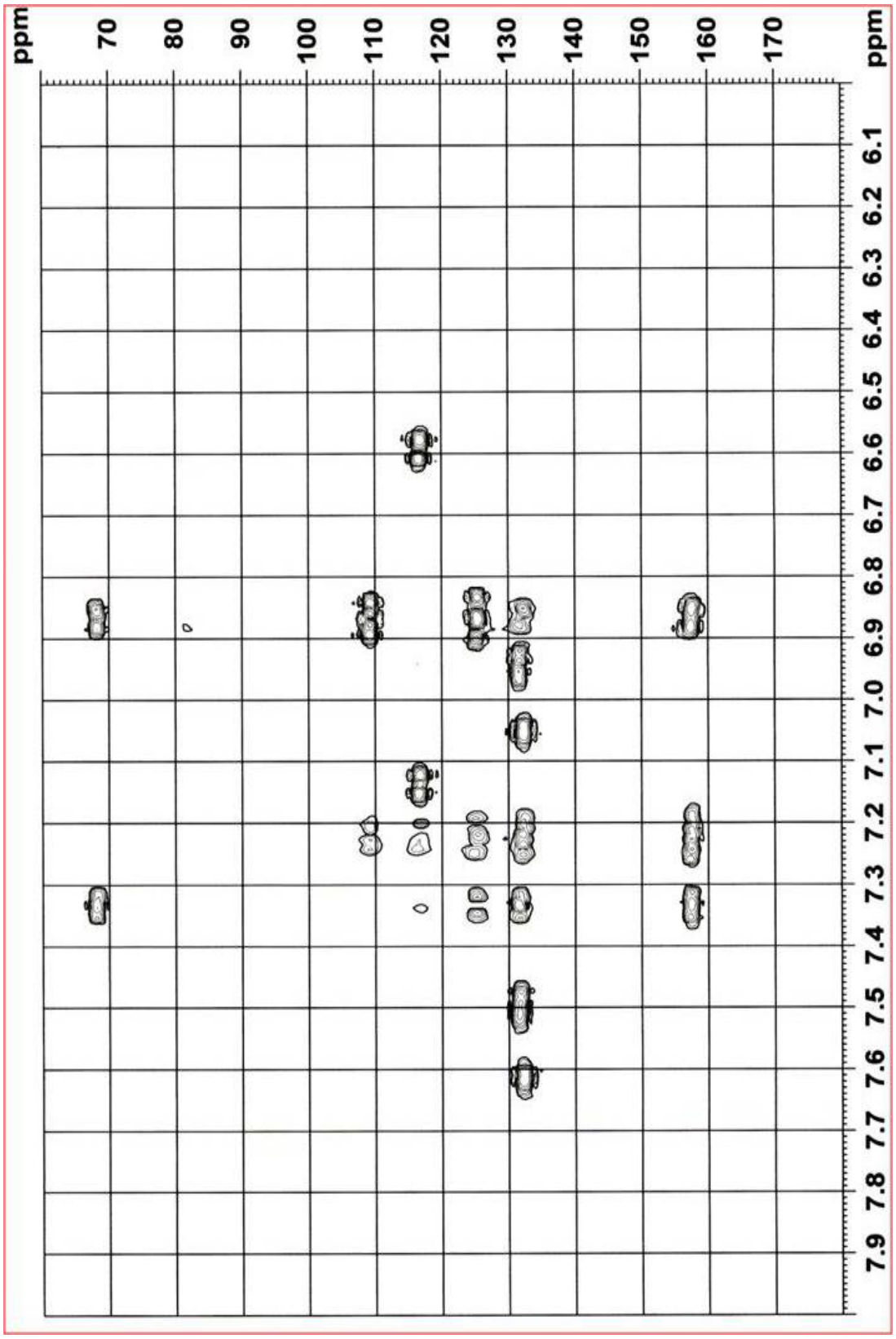

Fig. 3. 8. HMBC of the triyne.

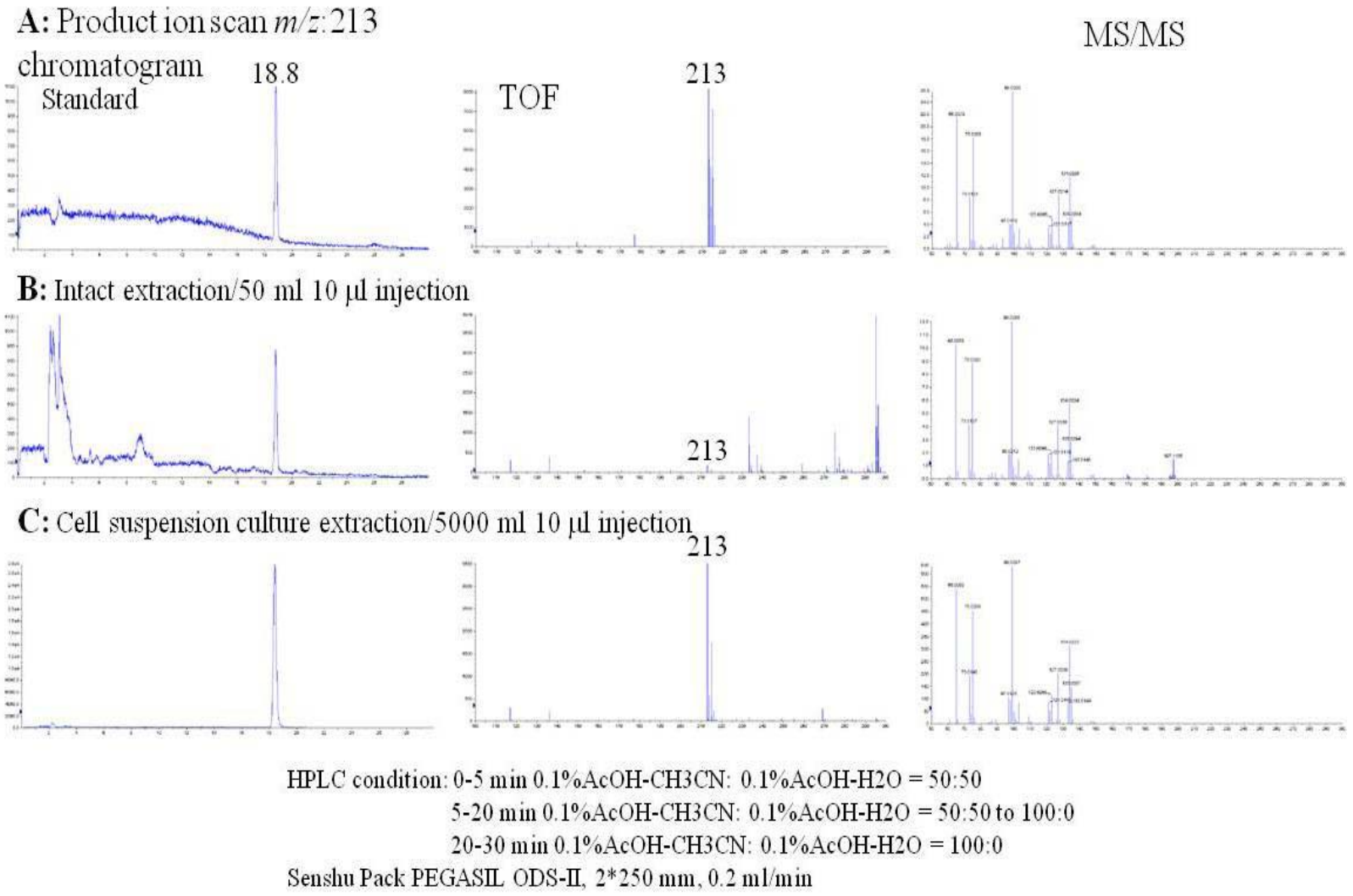

Fig. 3. 9. Detection of the triyne in different ethanolic extracts of *Helichrysum aureonitens*. **A:** Pure triyne as a standard; **B:** Intact plant **C:** Cell suspension cultures.

3.4.4 Instability of the triyne

On TLC analysis, the compounds were always visualized using UV light. After developing compound **1** on TLC plates and irradiating with UV light, a part of compound **1** was coloured to yellow by UV irradiation. Compound **1** was therefore also analyzed with two dimensional development on TLC (2D-TLC) followed by UV irradiation. Control plates were visualized by *p*-anisaldehyde. The result of the 2D-TLC elucidated that compound **1** from the cells of *H. aureonitens* cell suspension was decomposed to a yellow compound at the starting point with UV light (Fig. 3.10).

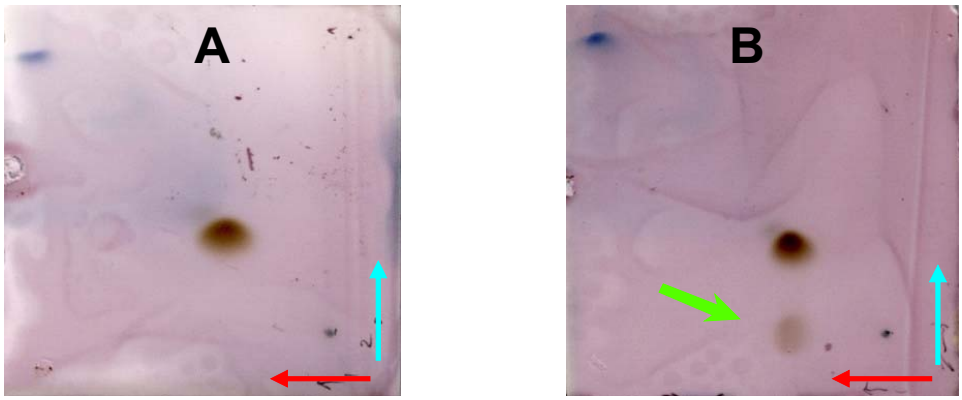

Fig. 3. 10. Two dimension thin layer chromatography (TLC) of the triyne. **A**: Control without UV light and emission with *p*-anisaldehyde; **B**: Degradation of the triyne after irradiation for 30 seconds with UV light after first TLC development. Green arrow shows the decomposed triyne by UV light. Narrow red and blue arrows show the direction of the first and second development respectively.

3.4.5 Bioassays

3.4.5.1 Antituberculosis activity

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the ethanol crude extracts of the cell suspension cultures against *M. tuberculosis* H37Rv were found to be 1.0 mg/ml and 2.0 mg/ml respectively. Compound **1** (the triyne) did not show antituberculosis activity at the highest concentration (200 µg/ml) tested. The MIC for the positive control "isoniazid" was found to be 0.2 µg/ml.

3.4.5.2 Toxicity and anticancer activity

The ethanol crude extracts of the cell suspension cultures and the triyne were evaluated *in vitro* for their cytotoxicity against Vero cells and the human prostate epithelial carcinoma (DU145) cell line. The triyne was about 8 times more toxic (IC_{50} value, 1.51 µg/ml) than the crude ethanol extracts (12.11 µg/ml) on a Vero cell line. The crude extracts and the triyne showed similar toxicity on a prostate cancer cell 4 line with IC_{50} values of 3.52 and 2.14 µg/ml respectively. The IC_{50} values of the positive control "Zeralenone" were found to be 1.63 and 0.53 µg/ml against Vero and DU145 cell lines respectively (Fig. 3.11).

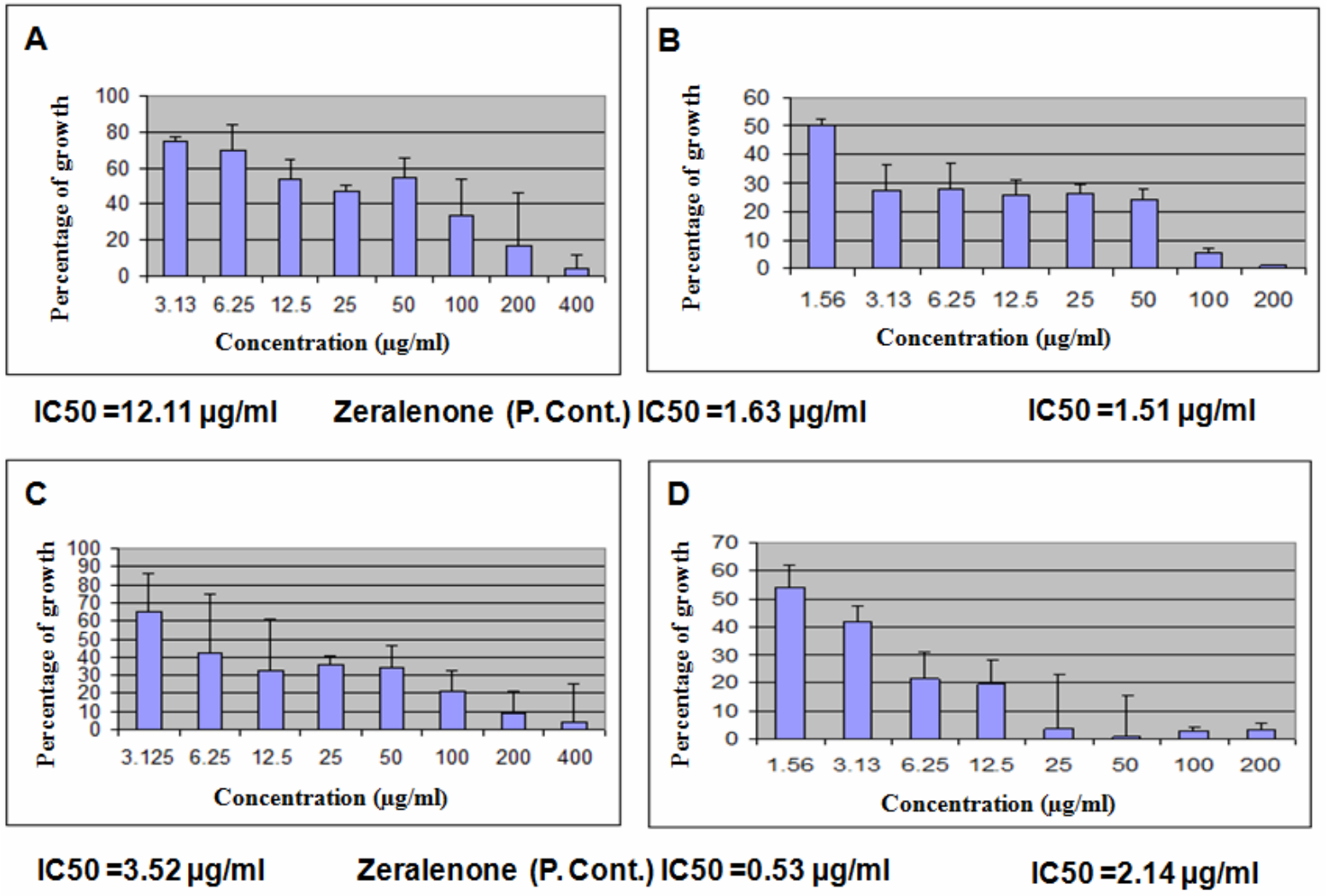

Fig. 3. 11. Effect of the ethanol crude extracts and the isolated the triyne (µg/ml) on the growth of Vero and DU145 cell lines. A: Crude extract (Vero cells); B: Chlorophenol triyne (Vero cells); C: Crude extracts (DU145); D: Chlorophenol triyne (DU145)

3.5 Discussion

The results of this part of the study regarding the production of the triyne in the *H. aureonitens* cell suspension cultures confirm what Bohlmann et al. (1984) found for the biosynthesis of the triyne in the roots of *H. tenuifolium*. They isolated the methyl ether derivative of the triyne and two other unusual chlorophenol acetylenes, helitenuin and helitenuone from the roots of *H. tenuifolium* (Fig. 3.12). They proposed that the isolated triyne could be synthesized from compounds **2** and **3** (Fig. 3.12), and the triyne would be the precursor of the compounds heliteniun and helitenuone **(**Bohlmann and Abraham, 1979). Based on this, it was proposed that the triyne is an intermediate in the acetylene biosynthetic pathway and the substrate for both helitenuin and helitenuone **(**Bohlmann et al., 1984). Although they were not able to isolate the triyne from the roots of *H. coriaceum,* the isolation of the triyne from the cell suspension cultures of *H. aureonitens* confirms their hypothesis.

The triyne was also detected in the aerial parts of the intact plants and the tissue cultured plants of *H. aureonitens* by LC-MS analysis, but at very low concentrations (Fig. 3.9). This indicates the higher induction of the polyacetylenase enzyme under the cell suspension conditions. This response of the cells in the cultures could have been driven by the exogenously applied phytohormones through up-regulating the involved genes in the triyne production biosynthetic pathway. Bohlmann (1984 and 1979) proposed the presence of the triyne in the roots (which normally grow in darkness) of *H. coriaceum* and *H. tenuifolium*. In our experiment the triyne was produced in the *H. aureonitens* cell suspension cultures grown in dark conditions. It might therefore be that dark conditions are necessary for the induction of the triyne, but more experimentation is needed to confirm this. This was confirmed in another

experiment in which the triyne could not be detected after 72 hrs exposure of the cell cultures to UV light (data not shown).

Two biosynthetic pathways are proposed for the production of acetylenic compounds including fatty acids and polyketide (Bohlmann et al., 1984 and Paiva, 2000). In the fatty acids pathway, the acetylenes are formed by the condensation of "activated" acetate units supplied either as malonyl CoA or acetyl CoA or from common fatty acids like linoleic acid. Polyacetate molecules can also be synthesized by specific "polyketide synthases" and modifying enzymes (Paiva, 2000). Originally the fatty acids were proposed as the starting point in the biosynthesis of linear polyacetylenic compounds, in contrast the poliketide biosynthetic pathway has also been proposed for the aromatic polyacetylenic compounds, e.g. the triyne can be biosynthesized from this pathway as well as phloroglucinols. The presence of phloroglucinols in the genus *Helichrysum* supports the contribution of the poliketide enzymatic system in the production of acetylenic compounds in this genus. However, because we could not isolate any intermediate related to the triyne we were not able to indicate which biosynthetic pathway is driving the chlorophenoltriyne production.

Antituberculosis activity has also been reported in other *Helichrysum* species. The MIC for example of the acetone extracts of *H. melanacme* and *H. odorastissimum* were found to be 0.50 mg/ml whereas the acetone extracts of *H. caespititium* exhibited an MIC of 0.10 mg/ml (Lall and Meyer 1999; Meyer et al., 2002). It has been shown that the higher activity of crude extracts when compared to the pure component is due to the interaction between different molecular components, and this is required for the optimal therapeutic effect of the plant extracts (Raskin et al., 2002). In root extracts of *Tripterygium wilfordii*, the main active ingredient identified was a triptolide, a traditional Chinese medicine to treat rheumatoid

arthritis, is shown to be toxic unless given as a part of the root extract, suggesting that other unidentified extract components increase its safety and, possibly, efficacy (Su et al., 1990).

The anticancer activity of some acetylenic compounds has already been reported (Siddiq and Dembitsky, 2008). Polyacetylenes found in ginseng (Matsunaga et al., 1990) and other medicinal plants (Jung et al., 2002) have been reported to exhibit anticancer activity. There are also some reports on the anticancer activities in the *Helichrysum* genus. Extracts from *H. pallassi, H. armenium, H. plicatum* have been reported to have considerable inhibition on DNA topoisomerase I. The inhibitors of DNA topoisomerase I represent a major class of anticancer drugs (Kucukogola et al., 2006).

Results obtained from the TLC analysis of the ethanol extracts showed that there are no observable flavonoid compounds in the *H. aureonitens* cell suspension cultures. This result was confirmed by GC-MS and LC-MS analysis. Some flavonoids including quercetin and galangin were detected in the ethanolic extracts of intact plants. This result was confirmed by LC-MS analysis.

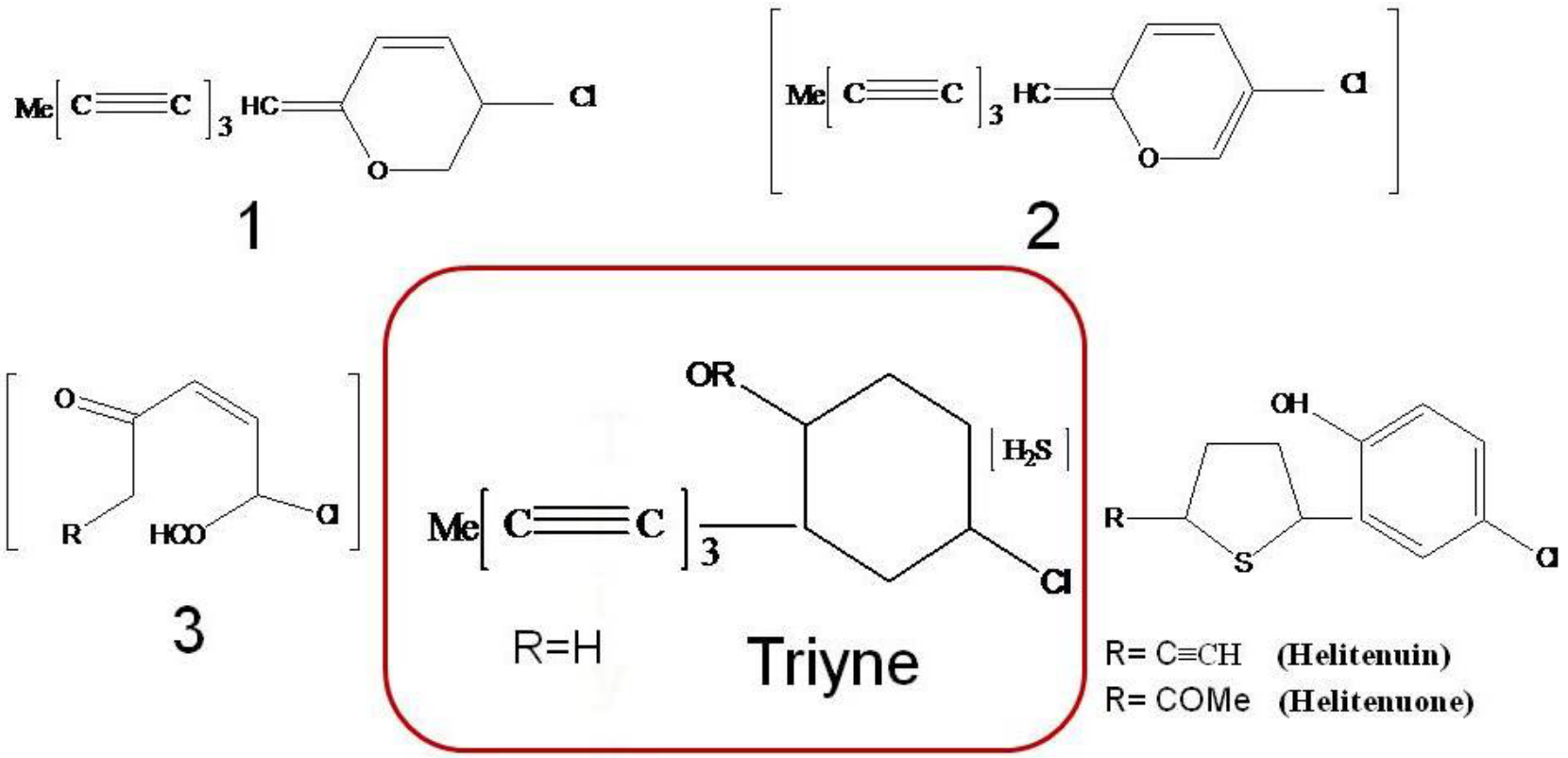

Fig. 3. 12. Proposed biosynthetic pathway for the chlorophenol compounds in *Helichrysum coriaceum***.** The boxed compound shows the position of the proposed the triyne in the biosynthetic pathway of the chlorophenol compounds. Bohlmann et al., 1984.

3.6 Conclusions

As mentioned before, one of the initial objectives for this study was the enhancement of galangin in cell suspension cultures of *H. aureonitens*. The results of this project showed that galangin is not produced in the cell suspension culture system. In fact none of the detected flavonoids in the intact plants were present in the cell suspension cultures of *H. aureonitens.* Instead there were two other compounds detected in a high concentration of which only compound **1** was isolated, purified and identified as a new chlorophenol compound. The new compound was identified as 4-chloro-2-(hepta-1,3,5-triyne-1-yl)-phenol. According to the chemical structure and the biosynthesis of the triyne the possibility of a direct relationship between it and the galangin pathway was rejected. Flavonol compounds e.g. galangin are light dependent compounds, while the triyne was detected in roots or in cell suspension cultures, which normally grow in dark conditions.

According to the results of this chapter, it could also be concluded that the triyne is a novel chlorophenol compound in the genus *Helichrysum* isolated for the first time from *H. aureonitens* cell suspension cultures. The triyne is perhaps the missing link in the chlorophenol compounds' biosynthetic pathway in *Helichrysum spp,* which was proposed by Bohlmann et al. (1984). Another interesting characteristic of the triyne is the presence of a chlorine molecule in the chemical structure. The chlorinated phenolic compounds are hardly found in nature. Results obtained at tested concentrations of the triyne against *M. tuberculosis*, showed no activity, but its toxicity against Vero cells was considerable when compared to the crude extracts. This indicates that the potential benefits of the triyne might be in anticancer activity.