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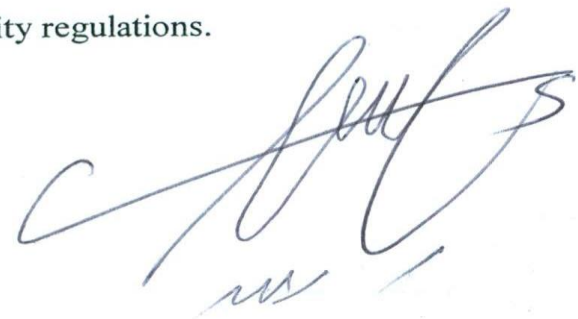

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Date:

Fungal Transformational Studies on β-Caryophyllene and Biological Evaluation of the Resulting Metabolites

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Thesis

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August, 2020

COMMITTEE DECISION

This Thesis (Fungal Transformational Studies on β-Caryophyllene and Biological Evaluation of the Resulting Metabolites) was successfully defended and approved on 19.8.2020

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DEDICATED

To my family and parents

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niger

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List of Abbreviations

$CDCl_3$	Deuterated chloroform
CD_3OD	Deuterated methanol
$CHCl_3$	Chloroform
MeOH	Methanol
NMR	**N**uclear **M**agnetic **R**esonance
COSY	**CO**rrelation **S**pectroscop**Y**
NOESY	**N**uclear **O**verhauser **E**ffect**S**pectroscop**Y**
HMQC	**H**eteronuclear **M**ultiple **Q**uantum **C**oherence
HMBC	**H**eteronuclear **M**ultiple **B**ond **C**oherence
IR	**I**nfra**r**ed
UV	**U**ltra**v**iolet
ESI-MS	**E**lecto**S**pray **I**onization-**M**ass **S**pectra
HRESI-MS	**H**igh **R**esolution **E**lecto**S**pray **I**onization -**M**ass **S**pectra
J	Coupling constant
s	**s**inglet
d	**d**oublet
t	**t**riplet
q	**q**uartet
dd	**d**oublet of **d**oublet
Hz	**H**ert**z**
g	**g**ram
L	**L**iter
DEPT	**D**istortionless **E**nhancement by **P**olarization **T**ransfer
MHz	**M**ega **H**ert**z**
nm	**n**ano**m**ete**r**
mm	**m**illi**m**eter
mL	**m**illi**L**iter

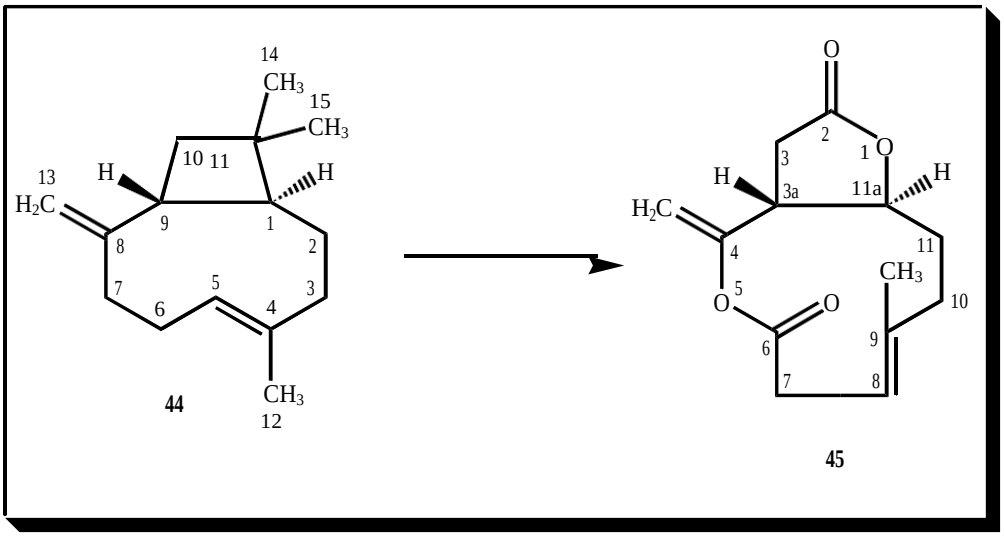
IC50	**Half Maximal Inhibitory Concentration**
2D-NMR	**Two D**imensional **N**uclear **M**agnetic **R**esonance
TLC	**T**hin **L**ayer **C**hromatography
TMS	**T**etra **M**ethyl **S**ilane
δ	**Chemical shift in ppm**

Summary

Microbial transformations are organic reactions, catalyzed by microorganisms such as fungi. They are often the key steps in the synthesis of many drug molecules, and natural products and their derivatives. This technique has also been widely employed for the synthesis of new analogues of terpenoids. During this work, a comparative microbial transformational study of selected terpenoid (β-caryophyllene) was carried out by various fungi to produce a new metabolite with interesting biological activities. The transformed product was purified by using chromatographic procedures, and its structure was elucidated with the help of the spectroscopic techniques. The transformed product was also evaluated for their biological activities, such as antioxidant and β-carotene bleaching assays.

β-Caryophyllene ((1R,4E,9S)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0]undec- 4ene, $C_{15}H_{24}$) (**1**), a bicyclic sesquiterpene isolated from the essential oils of the stems and the flowers of *Syzgium aromaticum* (cloves), and the essential oils of *Cannabis sativa* and *Salvia rosmarinus* (rosemary), has been shown to have anti-cancer, antioxidant, anti-inflammatory and anti-bacterial activities.

Microbial transformation of β-caryophyllene (**44**), by suspended cell-cultures of the plant pathogenic fungus *Aspergillus niger*, resulted in the production of one metabolite. The metabolite was identified as (3a,11a,E)-9-methyl-4- methylene3a,4,7,10,11,11a-hexahydro-2H-furo[3,2-c]oxecine-2,6(3H)-dione (**45**), by different spectroscopic methods. Compound **2** was found to be new.



The antioxidant activity of β-caryophyllene (**44**), metabolite **45** and the standard was also studied on the basis of the radical scavenging effect of the stable DPPH free radical. Compound **45** showed appreciable antioxidant activity.

Title of The Thesis

Fungal Transformational Studies on β-Caryophyllene and Biological Evaluation of the Resulting Metabolites

Introduction

Biotransformation

Biotransformations are organic reactions catalyzed by the biological catalysts. Biological catalysts can either be pure enzymes or microbial cultures (Hanson, 1995; Choudhary, *et al*., 2003; Choudhary, *et al*., 2003).

Biotransformations can be divided into two different classes. The xenobiotic biotransformations, which include the biotransformation of substrates that are totally alien to the particular system. The biosynthetically-directed transformations, involve a formal relationship between the substrate and the natural biosynthetic intermediate. There are many advantages of biotransformations as compared to chemical synthesis. For instance, biotransformations are often regio-, stereo-, and enantio-specific, and have the capacity to produce chiral compounds from racemic mixture. Moreover, the reaction conditions of biotransformation are almost neutral and they require no protection of functional groups present at the substrate. Other advantages of biotransformation include regiospecificity which is their ability to produce transformations at centers which are chemically unreactive. In addition to that, biotransformations can be commercially feasible and more direct as compared to their chemical synthesis analogs. They can be performed under conditions that are usually regarded as environmentally friendly.

Fungal transformation

Microbial transformation is only applied in the transformations of organic compounds by using whole cell cultures of microorganisms, such as fungi. A variety of organic reactions can be catalyzed by fungi. Microorganisms can be employed in the production of value-added compounds from cheaper raw materials. They often tend to

produce more than one metabolite. Microorganisms are often cheaper than the isolated enzymes.

Fungal transformation ofterpenoids

Microbial transformations by fungi have been applied for the conversion of a variety of organic compounds, especially terpenoidal compounds, since it is difficult to carry out direct chemical changes on unreactive carbon centers of terpenoidal molecules by conventional chemical reactions.

Terpenes are natural hydrocarbons. They are biosynthesized by the combinations of isoprene units. Terpenoids are usually modified terpenes, where a particular carbon is oxidized, or methyl group is re-arranged or removed. Terpenes are divided into different classes based on the number of carbon atoms in their skeleton. These include monoterpenes, sesquiterpenes, diterpenes, sesterterpenes, triterpenes and tetraterpenes containing ten, fifteen, twenty, twenty five, thirty and fourty carbon atoms, respectively. Microorganisms such as fungi have been applied extensively in the structural conversions of terpenoids with high regio- and stereo-selectivity (Choudhary, Batool, Khan, Sultana, Shah, and Atta-ur-Rahman, 2008; Oliveira, Filho, and Leal, 2005; Aleu, H-Galan, Hanson, Hitchcock, and Collado, 1999; Ardekani, Linley, Harkiss, Mohagheghzadeh, Gholami, and Mosaddegh, 2007).

For instance, the fungal transformation of the fungistatic sesquiterpenoid (**1**) by the plant pathogen *Botrytis cinerea* has yielded hydroxy derivatives **2-3** as the major and oxo derivatives **4-5** as minor metabolites (Figure-1). In the course of this work, some changes in the growth of *B. cinerea* were observed. Firstly, the growth of the fungus was inhibited when the substrate was added to the broth. Secondly, a careful examination of the broth extracts, after the biotransformation, showed that botrydial

and its derivatives were not produced during the period in which the substrate was present in the broth (Aleu, H-Galan, Hanson, Hitchcock, and Collado, 1999).

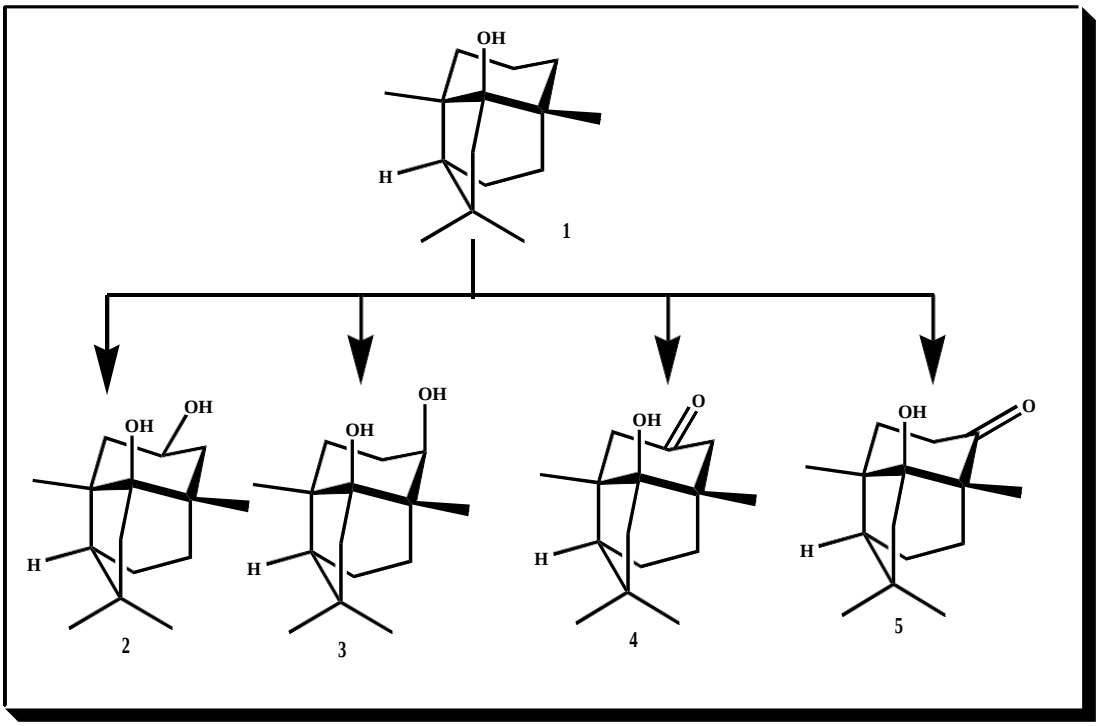


Fig. 1: Biotransformation of the fungistatic sesquiterpenoid, ginsenol (**1**), by *Botrytis cinerea*.

The sesquiterpene ester, ferutinin (**6**) was subjected to transformation, by suspended fungal cultures of *Aspergillus niger*, *Rhizopus stolonifer*, and *Fusarium lini*. These fungi produced only one metabolite, identified as ferutinin α-epoxide (**7**) (Fiqure-2) (Choudhary, M.I., et al., 2013). The metabolite showed a good antibacterial activity against *Staphylococcus aureus*, comparable to the substrate ferutinin (**6**).

A diterpenoidal type compound, clerodane lactone (**8**), was subjected to fungal transformation by using *Rhizopus stolonifer* (Choudhary, M.I., et al., 2013). This led to the production of 15, 16-dihydroxyclerodane lactone (**9**) and 2α-hydroxyclerodane lactone (**10**) (Figure-3). The structures of metabolites were elucidated with the help of

spectroscopic techniques. The antibacterial activities of clerodane lactone (**8**) and its metabolites **9-10** against the Gram-positive and Gram-negative pathogenic organisms were also studied. They showed a moderate activity against these pathogenic organisms. On the other hand, fungal transformation of clerodane methyl ester (**11**) by

R. stolonifer yielded clerodane methyl ester-15-one (**12**), clerodane methyl ester-16- one (**13**), 16-hydroxyclerodane methyl ester-15-carboxylic acid (**14**), and 15- hydroxyclerodane methyl ester-16-carboxylic acid (**15**) (Figure-4) (Choudhary, M.I., et al., 2013). The structures of the transformed products were deduced with the help of the spectroscopic techniques. The antibacterial activity of clerodane methyl ester, and its metabolites was also evaluated. These derivatives showed a good activity against both Gram-negative and Gram-positive organisms.

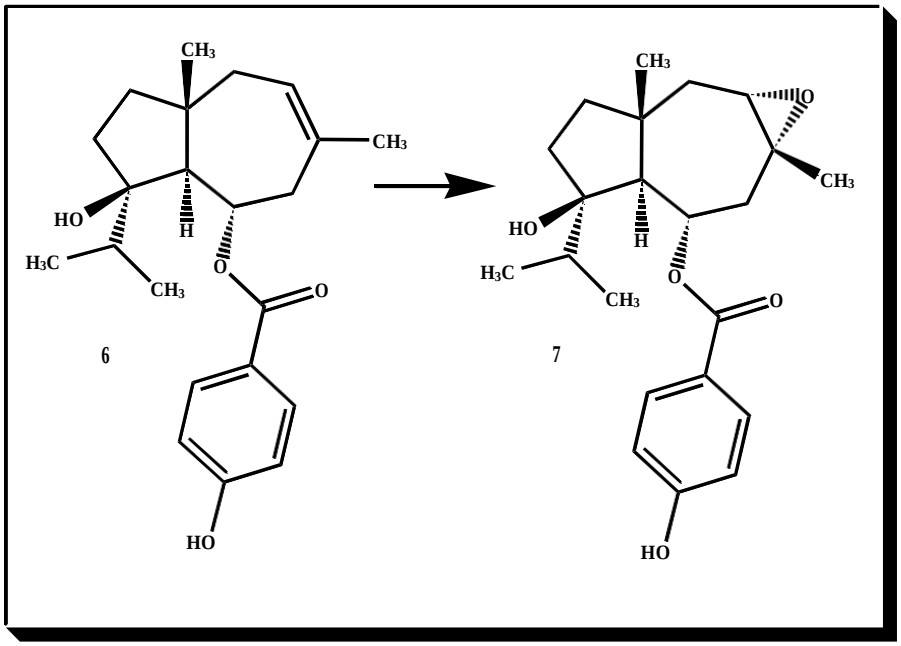


Fig. 2: Biotransformation of ferutinin (**6**) by *A. niger*, *R. stolonifer*, and *F. lini.*

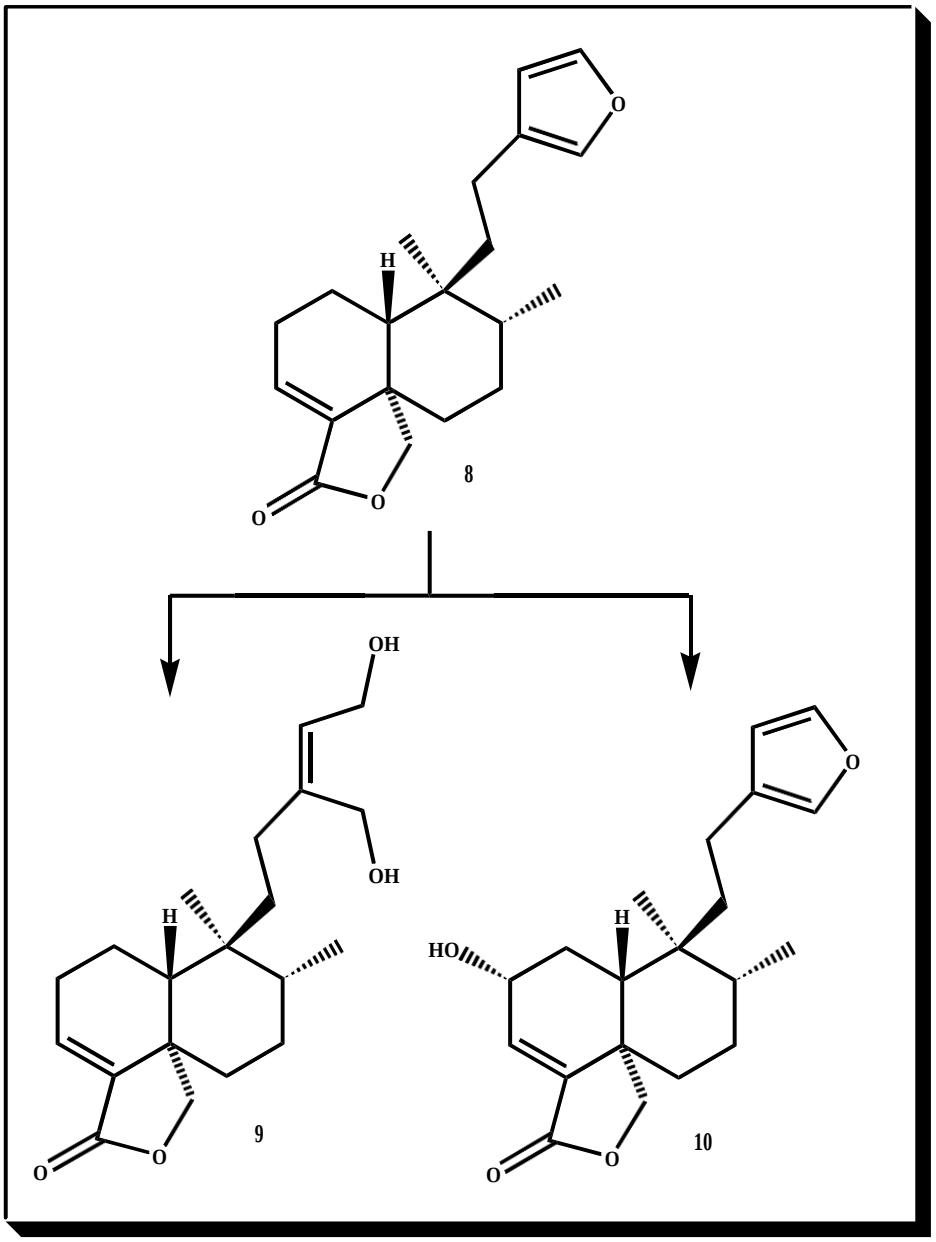


Fig. 3: Biotransformation of clerodane lactone (**8**) by *Rhizopus stolonifer*.

Microbial transformation of the monoterpenoid; thymoquinone (**16**) by suspended cell-cultures of the plant pathogenic fungus *Aspergillus niger* resulted in the production of three metabolites **17-19** (Figur-5). These metabolites were identified as 5-isopropyl-2-methyl-2,4-cyclohexenone lactone (**17**), hydroxythymoquinone (**18**), and 4-hydroxy-2-isopropyl-5-methylphenol (**19**) by different spectroscopic methods

(Mohammad, M.Y., et al., 2018). These metabolites showed potent antioxidant activity.

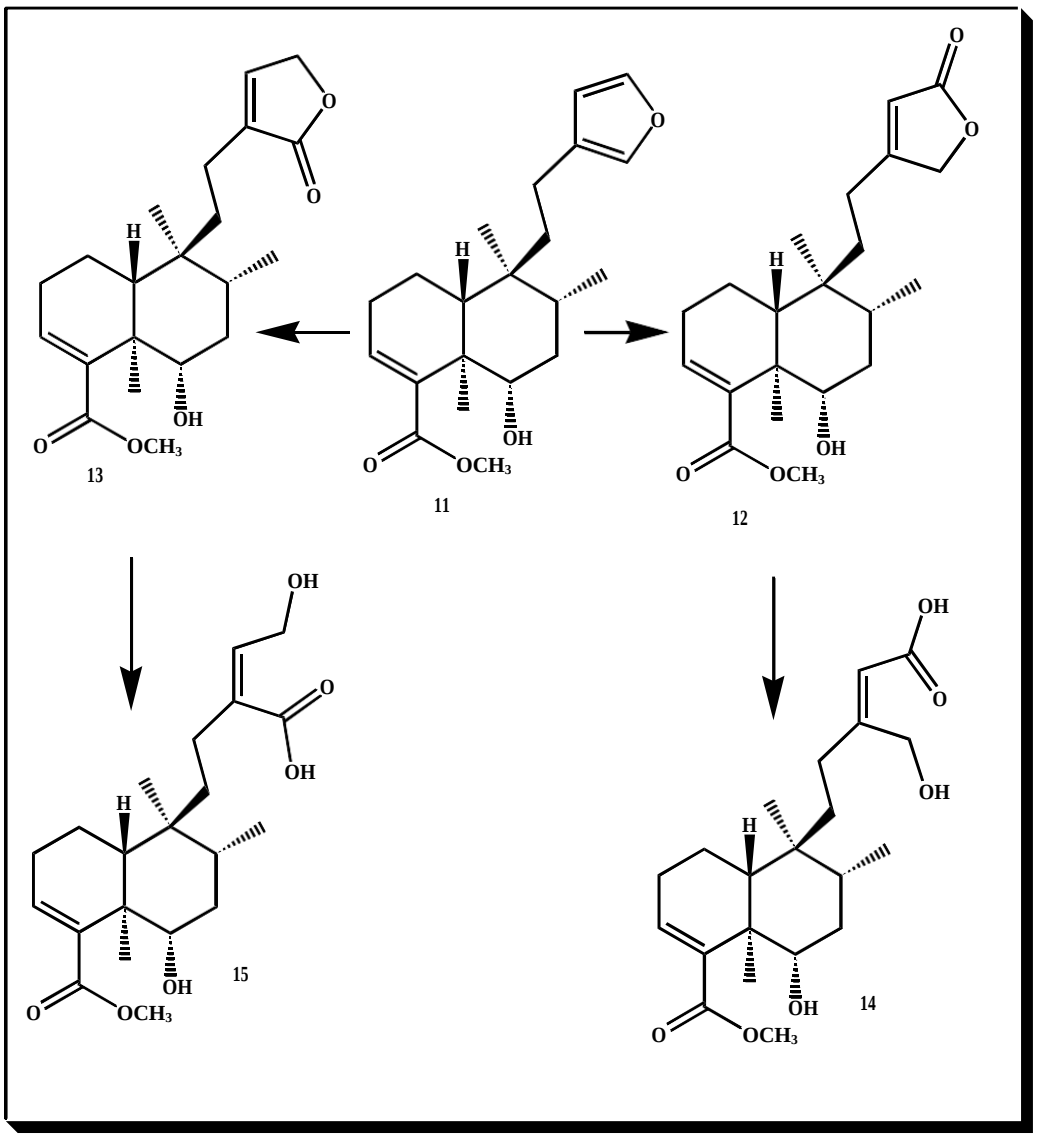


Fig. 4: Transformation of clerodane methyl ester (**11**) by *Rhizopus stolonifer*.

The oxidative and reductive capabilities of microorganisms have been known for a long time and are currently used in industrial reactions. Various classes of bioactive steroidal and terpenoidal compounds have been subjected to biotransformation to obtain more active and less toxic substances. Table-1 provides a comprehensive list of already biotransformed terpenoids along with their structures and literature references.

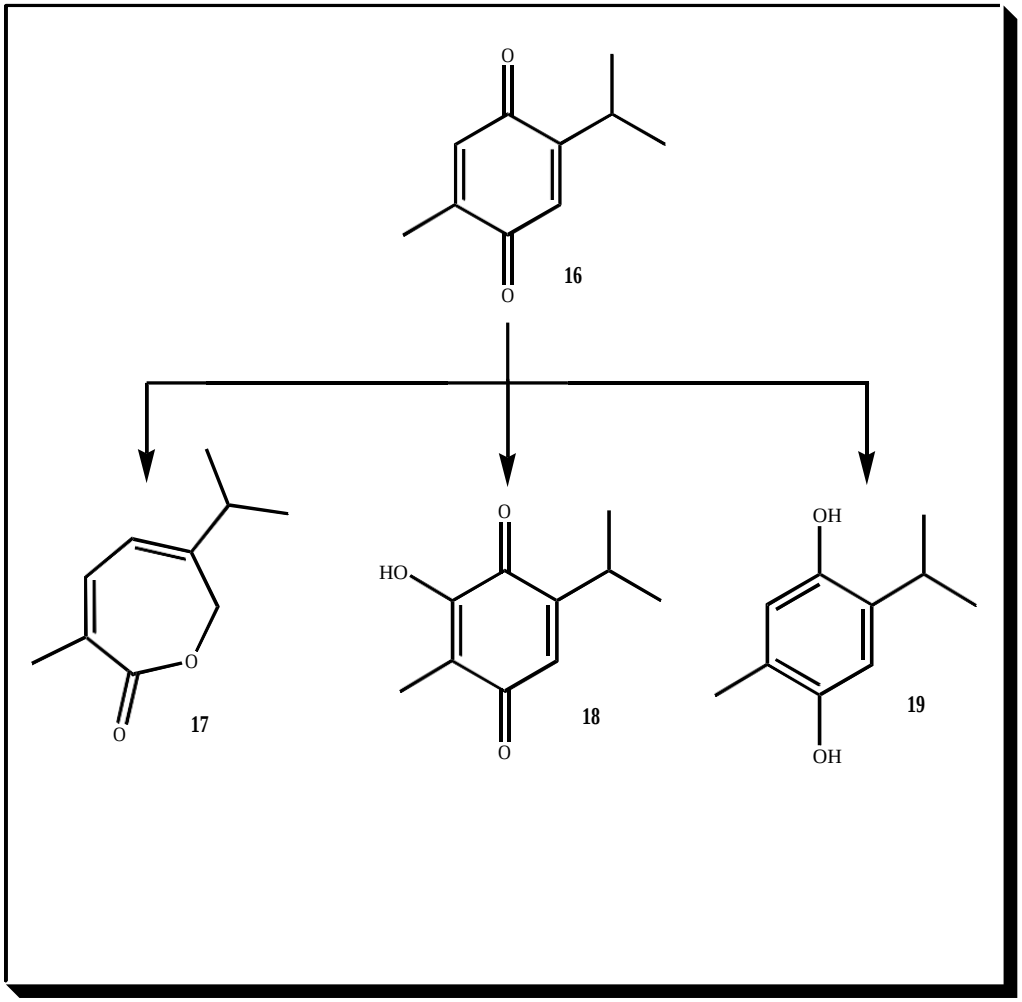
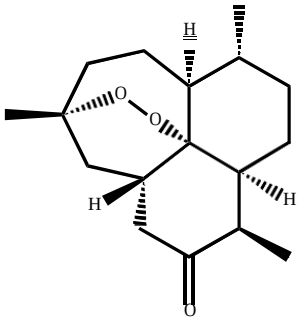
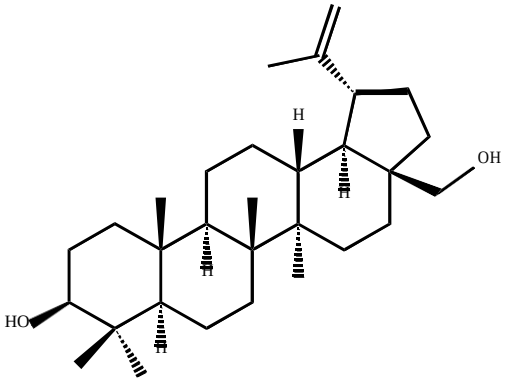
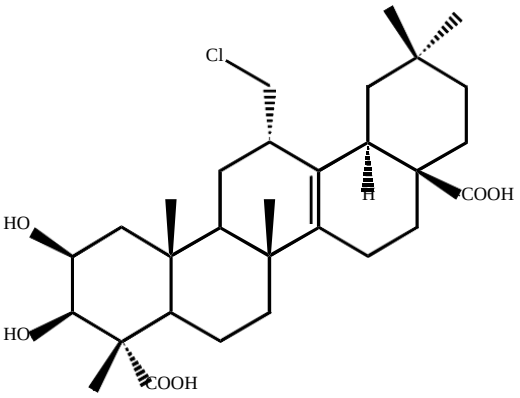
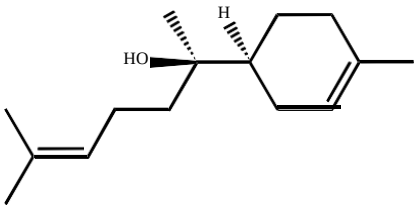
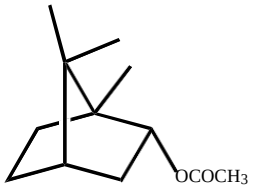
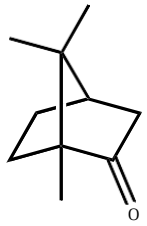
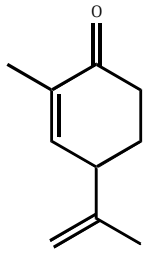
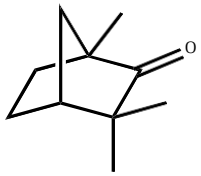


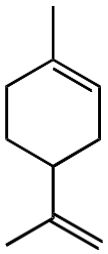
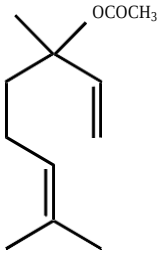
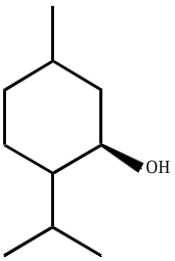
Fig. 5: Biotransformation of thymoquinone (**16**) by *Aspergillus niger*.

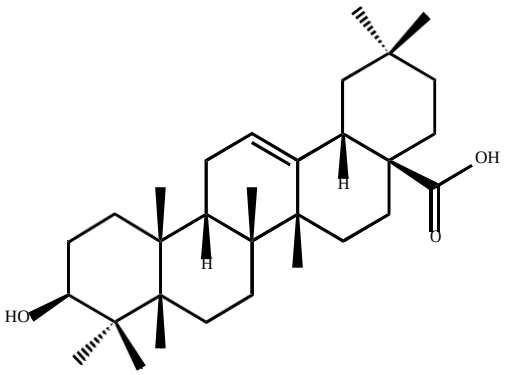
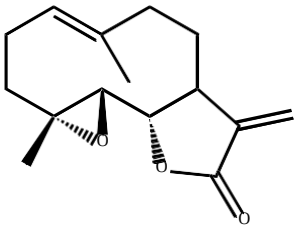
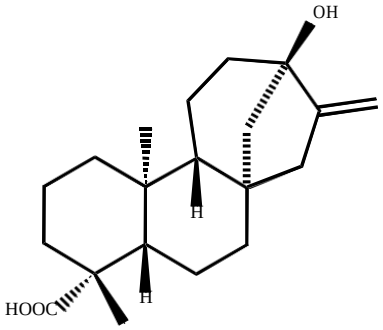
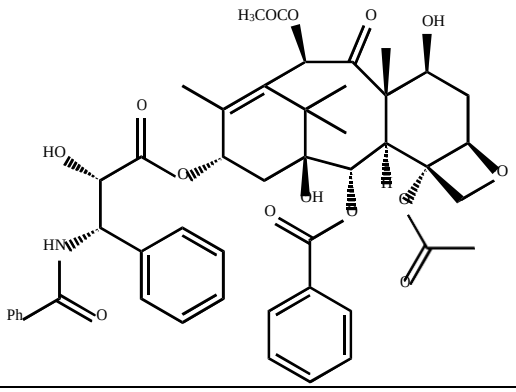
Table-1: List of known biotransformed terpenoids.

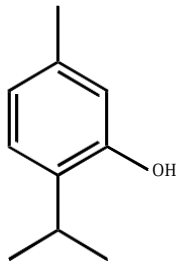
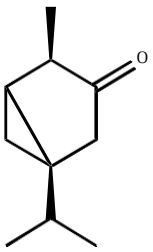
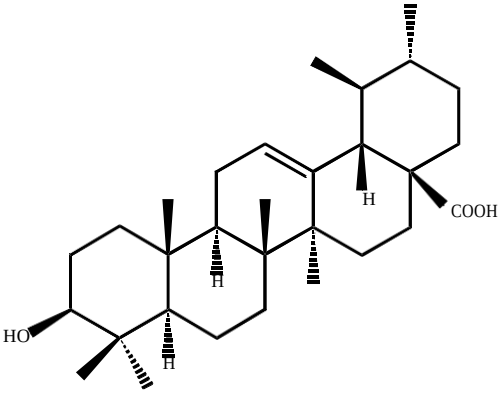
Compound	Chemical structure	References
Artemisinin (**20**)		(Lee, ElSohly, Croom, and Hufford,1989)

Betulin (**21**)		(Q-H Chen, Liu, H-F Zhang, G-Q He, and M-L Fu, 2009)
Betulonic acid (**22**)		(L-W Qian, Zhang, J-H Liu, and B-Y Yu, 2009)
(-)-α-Bisabolol (**23**)		(Miyazawa, Nankai, and Kameoka, 1995)
Bornyl acetate(**24**)		(Ardekani, Linley, Harkiss, Mohagheghzad eh, Gholami, and Mosaddegh,

		2007)
(-)-Camphor (**25**)		(Miyazawa, and Nakahashi, 2011; Orihara, Noguchi, and Furuya, 1994)
Carvone (**26**)		(Hamada, Yasumune, Fuchikami, Hirata, Sattler, Williams, and Scott, 1997)
Fenchone (**27**)		(Orihara, and Furuya, 1994)

Limonene (**28**)		(Chatterjee, and Bhattacharyya, 2001; Cadwallader, Braddock, Parish, and Higgins, 1989)
Linalyl acetate(**29**)		(Ardekani, Linley, Harkiss, Mohagheghzad eh, Gholami, and Mosaddegh, 2007)
Menthol (**30**)		(Furuya, Orihara, and Miyatake, 1989)

Oleanolic acid (**31**)		(Choudhary, Batool, Khan, Sultana, Shah, and Atta-ur- Rahman, 2008)
Parthenolide (**32**)		(Galal, Ibrahim, Mossa, and El-Feraly,1999)
Steviol (**33**)		(Oliveira, Filho, and Leal, 2005)
Taxol (**34**)		(Chen, Li, Bollag, Y-c. Liu, andChang, 2001)

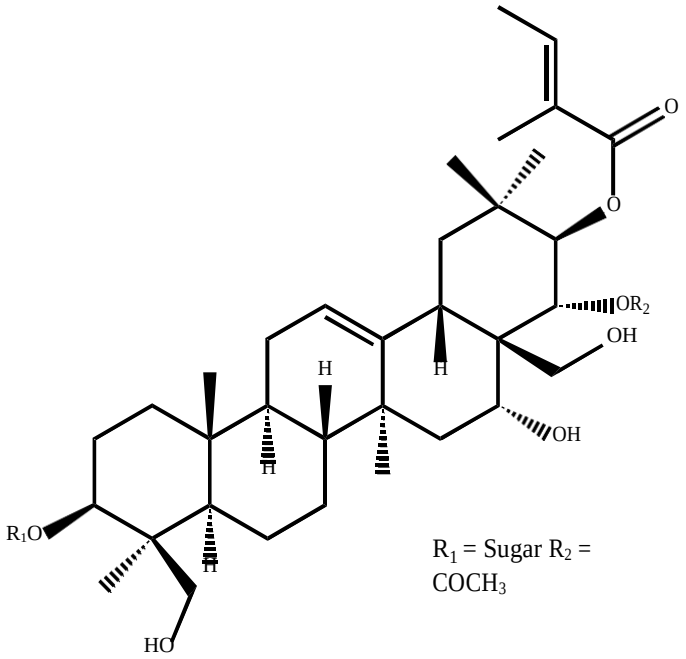
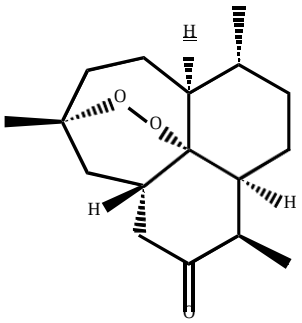
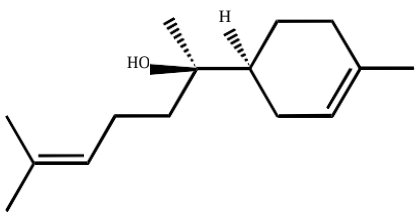
Thymol (**35**)		(Esmaeili, Khodadadi, and Safaiyan, 2012)
α-(−)-Thujone (**36**)		(Alaoui, Benjilali, and Azerad, 1994)
Ursolic acid(**37**)		(Fu, Yang,Cui, Feng, and Sun, 2011)

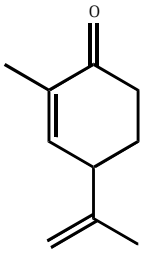
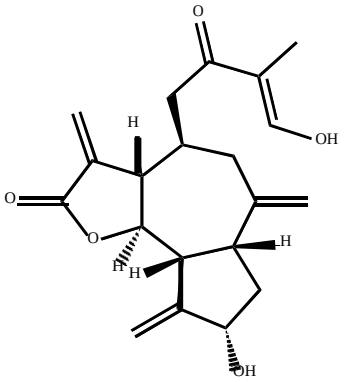
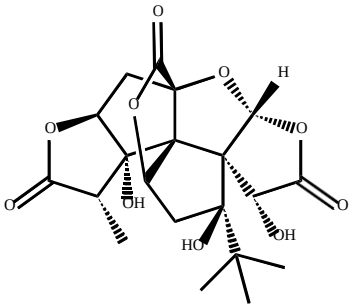
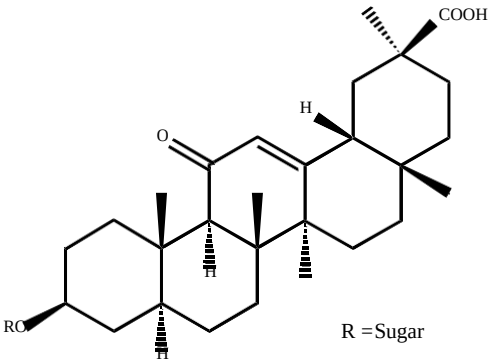
Terpenoidal drugs

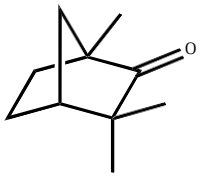
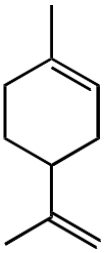
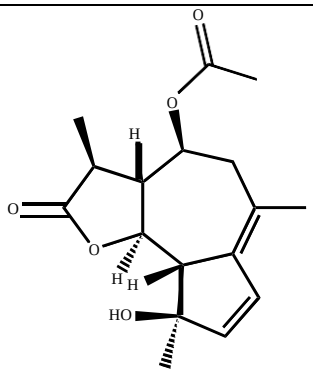
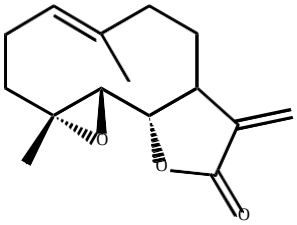
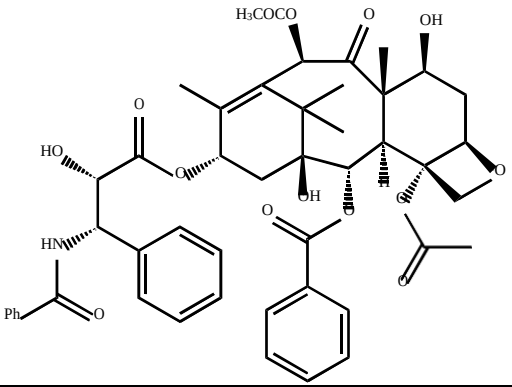
Terpenoids are classified as the largest group of secondary metabolites among all the natural compounds. Many terpenoidal compounds have therapeutic properties which are exploited for the treatment of many diseases. In 2012, the worldwide sales of terpenoidal drugs were approximately US $ 12 billion. Among these terpenoidal drugs, the anticancer drug taxol and the antimalarial drug artimesinin are also included. Other terpenoids have a wide range of biological activities against cancer,

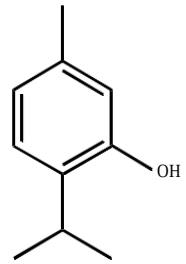
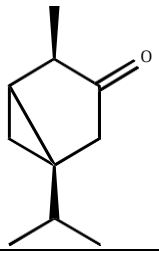
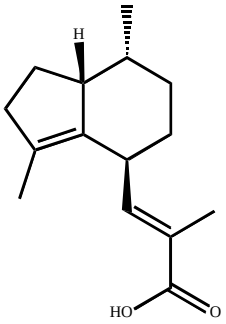
malaria, inflammation, and various viral and bacterial infections. Table-2 provides a list of terpenoidal drugs and their pharmacological uses.

Table-2: List of terpenoidal drugs and their pharmacological properties.

Terpenoidal drug	**Chemical structure**	**Pharmacological effect**
Aescin (**38**)	 R_1 = Sugar R_2 = $COCH_3$	Treatment of chronic venous insufficiency
Artemisinin (**20**)		Antimalarial
(-)-α-Bisabolol (**23**)		Anti-inflammatory and antiirritant

Carvone (**26**)		Carminative
Cynaropicrin (**39**)		Anti-hepatotoxic and cholesterol biosynthesis inhibitor
Ginkgolide A (**40**)		Treatment of dementia
Glycyrrhizin (**41**)		Antiulcer, anti-inflammatory, and expectorant
Fenchone (**27**)		Carminative

		
Limonene (**28**)		Used in aromatherapy
Matricin (**42**)		Ani-inflammatory and antispasmodic
Parthenolide (**32**)		Treatment of migraine
Taxol (**34**)		Anticancer
Thymol (**35**)		Used in aromatherapy, and antiseptic

		
α-(−)-Thujone (**36**)		Used in aromatherapy
Valerenic acid (**43**)		Hypnotics

General experimental methods

Microorganisms and culture medium

Microorganisms are purchased either from the American Type Culture Collection (ATCC) or from the Northern Regional Research Laboratories (NRRL). Microorganisms are grown on potato dextrose-agar or sabouraud glucose agar at 25

□C, and stored at 4 □C. The media for microorganisms differ from one organism to another, but generally the following ingredients are used in distilled H_2O: glucose, peptone, yeast extract, KH_2PO_4, glycerol, KCl, $MgSO_4.7H_2O$, and NaCl.

Fermentation and extraction conditions for substrate

The medium is distributed into conical flasks and then sterilized in autoclave at 121□

C. Mycelia are inoculated into all the flask media, and the flasks are placed in an incubator with rotary shaking at selected temperature for the growth of microorganism. After the complete growth of microorganism, substrate is dissolved in a particular organic solvent which is not toxic to microorganism, and then equally distributed to each cultural flask. The flasks are again placed on incubated shaker to allow the occurrence of fermentation. An additional flask labeled as a negative control, which contained a microorganism without substrate, is placed with the incubated flasks under the same conditions, and another additional flask, labeled as a positive control, which contained a substrate added to the medium without microorganisms, is also placed with the incubated flasks. The degree of transformation is checked every day by TLC, and compared with the controls. After the completion of fermentation, the mycelia are separated from the medium by filtration and then the medium is placed in a separatory funnel for extraction. The metabolites are extracted using a suitable organic solvent. This extraction is repeated three times. A drying agent is added to the organic extract to remove the suspended water. The crude extract containing the metabolites is collected by evaporating the organic solvent, using vacuum on rotavap, and then analyzed by TLC.

Isolation of transformed products

Different chromatographic techniques can be used to isolate the metabolites. The crude extract is adsorbed on silica and subjected to column chromatography. The metabolites are eluted and purified by solvent mixtures of different polarities.

Identification of transformed products

Structures of the metabolites are elucidated through comparative spectroscopic studies with the substrate (*i.e.* ^{1}H-NMR, ^{13}C-NMR, EI-MS, HREI-MS, DEPT-135^{o}, DEPT- 90^{o}, HMQC, HMBC, COSY, NOESY, FT-IR, UV, etc).

Biological activity screenings

The metabolites are evaluated for their bioactivities (i.e. enzyme inhibition, antioxidant, anti-bacterial, anti-fungal, anti-inflammatory, etc). The general experimental methods are summarized in figure-6.

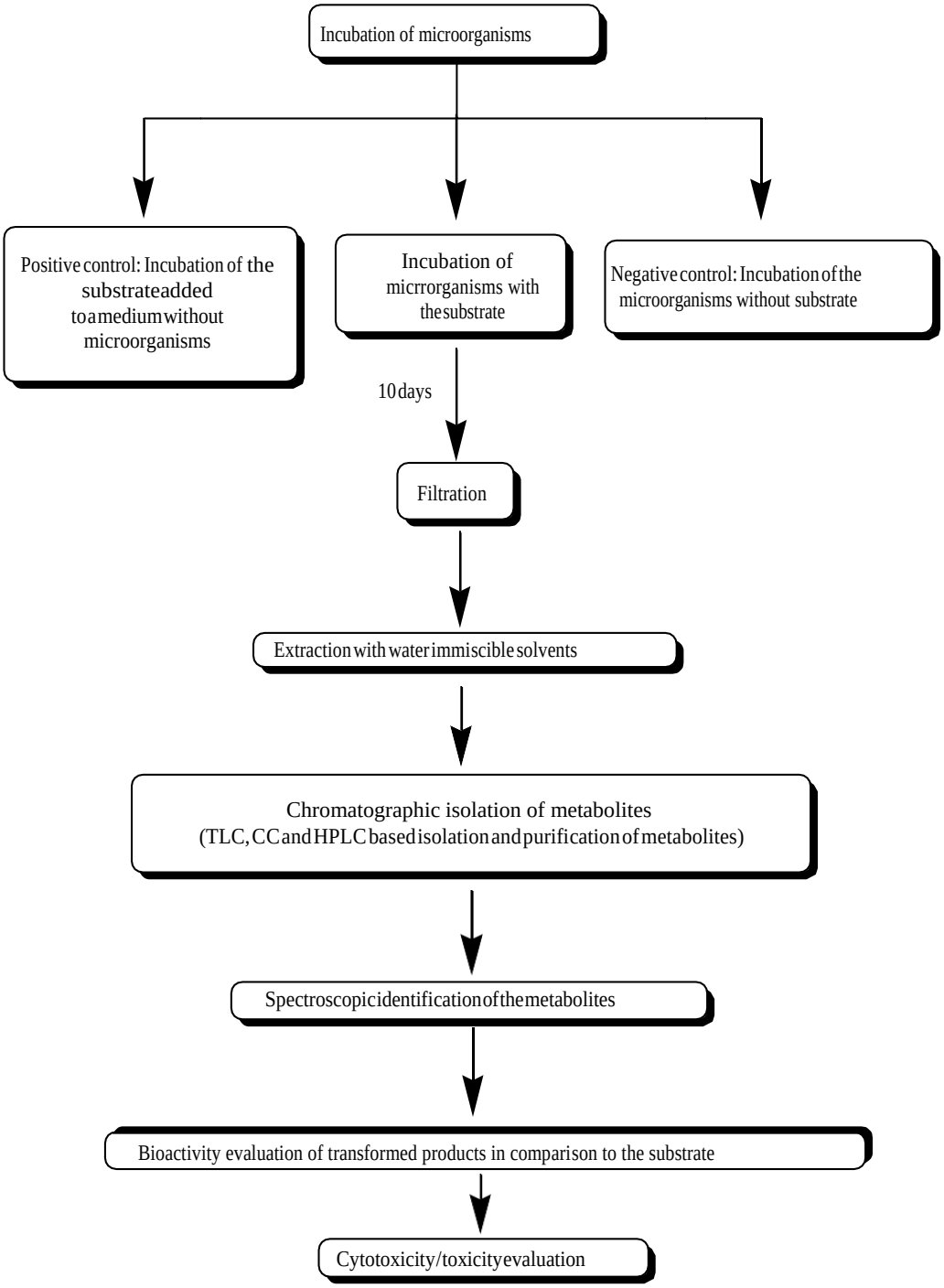


Fig. 6: Scheme of general experimental methods.

Objectives of the study

Terpenoids have many similarities in their structures. They are difficult substrates for a conventional chemical transformation. However, microorganisms can directly convert one carbon center only with high stereo-specifity by leaving other centers

unchanged. This is why biotransformation is mostly applied on terpenoids. For instance, there are many terpenoidal compounds (Choudhary, Musharraf, Sami, and Atta-ur-Rahman, 2004) which were subjected to biotransformation using different microorganisms.

Since biotransformation has been extensively applied on bioactive terpenoids (Choudhary, Ali Shah, Musharraf, and Shaheen, 2003; Choudhary, Musharraf, Nawaz, Anjum, Parvez, Hoong-Kun Fun, and Atta-ur-Rahman, 2005; Choudhary, Siddiqui, Musharraf, Nawaz, and Atta-ur-Rahman, 2005; Choudhary, Musharraf, Siddiqui, Khan, Azhar Ali, and Atta-ur-Rahman, 2005; Choudhary, Sultan, Hassan Khan, and Atta-ur-Rahman, 2005; Choudhary, Yousuf, Samreen, Ali Shah, Ahmed, and Atta-ur-Rahman, 2006; Devkota, Choudhary, Nawaz, Lannang, Lenta, Fokou, and Sewald, 2007; Faramarzi, Aghelnejad, Yazdi, Amini, and Hajarolasvadi, 2008; Farooq, Choudhary, Tahara, Atta-ur-Rahman, Husnu Can Baser, and Demirci, 2002; Farooq, Atta-ur-Rahman, and Choudhary, 2004; Choudhary, Siddiqui, Nawaz, and Atta-ur-Rahman, 2006; Atta-ur-Rahman, Yaqoob, Farooq, Anjum, Asif, and Choudhary, 1998), we decided to work on unstudied bioactive terpenoidal substrate, β-caryophyllene by biotransformation. The objective was to synthesize more active and less toxic new metabolites in comparison to substrate.

β-Caryophyllene (44) subjected to fungal transformation

The sesquiterpene, β-caryophyllene (**44**), $C_{15}H_{24}$ (Figure-7), was subjected to biotransformation using *Aspergillus niger* for the first time. β-Caryophyllene ((1R,4E,9S)-4,11,11-trimethyl-8-methylidenebicyclo[7.2.0]undec-4-ene, $C_{15}H_{24}$) (**44**), a bicyclic sesquiterpene isolated from the essential oils of the stems and the flowers of *Syzgium aromaticum* (cloves), and the essential oils of *Cannabis sativa* and *Salvia*

rosmarinus (rosemary), has been shown to have anti-cancer (Legault et al. 2007), anti-oxidant (Dahham et al. 2015), anti-inflammatory (Dahham et al. 2015) and anti- bacterial (Li Moo et al., 2020) activities.

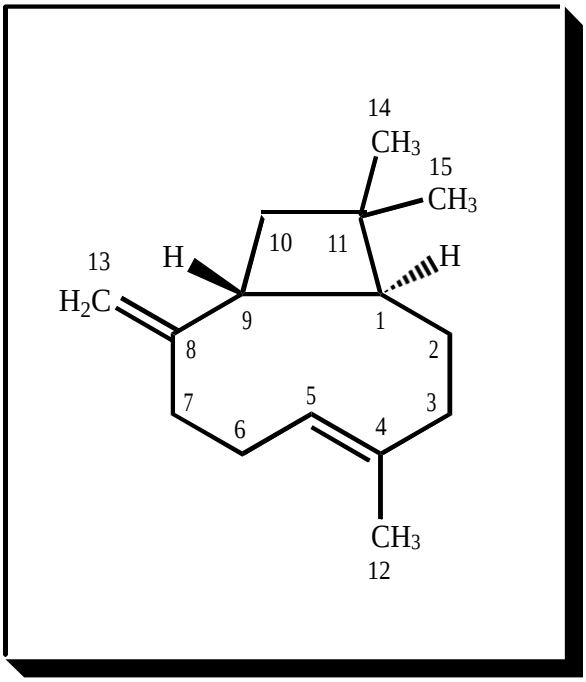


Fig. 7: The sesquiterpene; β-caryophyllene (**44**).

Experimental

General

β-Caryophyllene (**44**) was obtained from the sigma-aldrich. Silica gel precoated plates (Merck, PF_{254}; 20 20, 0.25 mm) were used for TLC. Silica gel (70-230 mesh, Merck) was used for column chromatography. Infrared (IR) spectra were recorded with an FT-IR-8900 spectrophotometer. ^{1}H- and ^{13}C- NMR spectra were recorded in $CDCl_3$ on a Bruker Avance-300 NMR spectrometer at 300 and 75 MHz, respectively,

with tetramethylsilane (TMS) as the internal standard. Standard pulse sequences were used for distortionless enhancement by polarization transfer (DEPT) and 2D-NMR experiments. The chemical shifts (values) were reported in parts per million, relative to TMS at 0 ppm. The coupling constants (*J* values) were reported in Hertz. High resolution mass spectrometry was performed using LC Mass Bruker Apex-IV mass spectrometer utilizing an electrospray interface.

Microorganism and culture medium

Aspergillus niger (ATCC 16404) was purchased from the American Type Culture Collection (ATCC), and grown on Sabouraud-4% potato dextrose-agar (Merck) at 28

C and stored at 4 C. The medium for *A. niger* of each substrate was prepared by mixing the following ingredients into distilled H_2O (3.0 L): glucose (60.0 g), peptone (15.0 g), yeast extract (15.0 g), KH_2PO_4 (15.0 g), and NaCl (15.0 g).

Fermentation and extraction conditions for compound 1

The fungal medium was transferred into 250 mL conical flasks (100 mL each) and autoclaved at 121 C. Mycelia of *A. niger* were transferred to all the flasks and incubated at 28 C for five days with rotary shaking (128 rpm). After five days, compound **44** (1.00 g, 4.9 mmol) was dissolved in 40 mL acetone and added to each flask (25 mg/ 1.0 mL acetone) and the flasks were placed on a rotatory shaker (128 rpm) at 28 C for fermentation. Parallel control experiments were conducted which included an incubation of the fungus without sample **44** and another incubation of **44** in a medium without fungus. Time course studies were carried out after every 24 hours and the degree of transformation was analyzed by TLC. After 7 days, the culture medium was filtrated and extracted with ethyl acetate (9 L) in three portions.

The extract was dried over anhydrous Na_2SO_4, evaporated under reduced pressure, and the brown gummy crude residue (1.5 g) was analyzed by thin layer chromatography.

Isolation of transformedproduct

The crude extract was dissolved in hexane: EtOAc (1:1 v/v), absorbed on silica (2.0 g) and subjected to column chromatography. The eluent system consisted of gradient mixtures of hexane and EtOAc. Compound **44** (120 mg) was eluted with hexane/EtOAc (9.5:0.5 v/v), while compound **45** (90 mg) was eluted in hexane/EtOAc (7.0:3.0 v/v).

β-Caryophyllene (**44**), white liquid. ^{1}H- and ^{13}C-NMR: Table-3.

(3aR,11aS,E)-9-Methyl-4-methylene-3a,4,7,10,11,11a-hexahydro-2H-furo[3,2-c]oxecine-2,6(3H)-dione (**45**), white liquid. IR (MeOH): 3060, 2935, 1752, 1646, 1185 cm^{-1}. HRESI-MS: *m/z* 237.11038 ($[M+H]^+$, $[C_{13}H_{16}O_4+H]^+$; calc. 237.11269).

^{1}H- and ^{13}C-NMR: Table-4.

DPPH free radical scavenging activity (Halub, et al., 2019)

The stock solutions of the test compound (1.5 mg/mL) were prepared in methanol. Stock solutions were serially diluted with the methanol to obtain lower dilutions (1.95-250, 5.47-700 and 0.5-32.0 µg/mL for **44**, **45** and ascorbic acid, respectively).

The free-radical scavenging activity of the samples were measured as a decrease in the absorbance of methanol solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH). A stock solution of DPPH (0.002 % w/v) was prepared in methanol and different concentrations of the samples were added (2–1000 µg/mL). Afterincubationatroom

temperature for 30 min, the pale pink color developed was measured at 517 nm using spectrophotometer and compared with the standard (1–100 µg/mL ascorbic acid). Free radical scavenging activity was expressed as the percentage inhibition calculated using the following formula:

$$\%\ \textbf{\textit{Free radical scavenging activity}} = \left[1 - \frac{Abs_{sample}}{Abs_{Control}}\right] \times 100$$

β-carotene bleaching (BCB) assay (Halub, et al., 2019)

A solution of β-carotene was prepared by dissolving 5 mg of β-carotene in 50 mL of chloroform. An aliquot of the 3 mL was added to 40 mg linoleic acid and 400 mg of tween 40. It was mixed and set aside for 2 minutes. The chloroform was evaporated off using nitrogen gas. The residue was reconstituted in 100 mL of distilled water using vortex. Immediately after preparation the absorbance of this solution was recorded at 470 and 700 nm. Different solutions of samples (25 µg/mL to 1000 µg/mL) were prepared in methanol (with the aid of 0.05% Tween-40). β-Carotene- linoleic acid emulsion (1mL) was mixed with different solutions of oil (0.25 ml). All the solutions (control and test) were capped and incubated (50°C) for 1 hour. The control sample contain equivalent amount of methanol (0.05% Tween-40). The absorbance of the solutions (λ_{470} and λ_{700} nm) was determined after 60 min. All determination was carried out in triplicate; the degradation rate (DR) and antioxidant activity was calculated.

Degradation rate (DR) of β-carotene = Ln ($A_{initial}/A_{sample}$)/60 Antioxidant

activity (%)= $$\left[1 - \frac{Degradation\ rate\ of\ sample}{Degradation\ rate\ of\ control}\right] \times 100$$

Table-3: ^{13}C-NMR and ^{1}H-NMR data of β-caryophyllene (**44**) (300 MHz; $CDCl_3$).

C. No.	^{13}C-NMR chemical shifts	^{1}H-NMR chemical shifts
1	53.7 (*d*)	1.96 (1H. m)
2	29.1 (*t*)	1.17 (1H, m), 1.42 (1H. m)
3	45.5 (*t*)	1.91 (1H, m), 2.01 (1H. m)
4	135.1 (*s*)	-
5	124.6 (*d*)	5.20 (1H, m)
6	27.8 (*t*)	1.99 (1H, m), 2.02 [a] (1H. m)
7	34.9 (*t*)	1.95 (1H, m), 2.05 [a] (1H. m)
8	154.6 (*s*)	-
9	48.5 (*d*)	2.63 (1H, m)
10	40.3 (*t*)	1.75 (1H, m), 2.00 [a] (1H. m)
11	33.9 (*s*)	-
12	16.3 (*q*)	1.82 (3H, s) 0.99 (3H, s)
13	111.7 (*t*)	4.92 (1H, s), 5.11 (1H, s)
14	26.3 (*q*)	0.99 (3H, s)
15	26.3 (*q*)	0.99 (3H, s)

Multiplicities were determined by DEPT experiments.

[a] These values are interchangeable.

Table-4: ^{13}C-NMR and ^{1}H-NMR data of metabolite **45** (300 MHz; $CDCl_3$).

C. No.	^{13}C-NMR chemical shifts	^{1}H-NMR chemical shifts
1	72.4 (*d*)	3.79 (1H, m)
2	31.3 [b] (*t*)	1.29 (1H, m), 1.58 (1H. m)
3	31.6 [b] (*t*)	1.85 (1H, m), 2.06 (1H. m)
4	137.5 (*s*)	-
5	129.8 (*d*)	5.20 (1H, dd, *J* = 9.8, 4.9 Hz)
6	30.2 [b] (*t*)	2.12 [c] (1H, m), 2.31 (1H. m)
7	171.0 (*s*)	-
8	139.1 (*s*)	-
9	47.0 (*d*)	3.39 (1H, m)
10	29.3 (*t*)	1.92 (1H, m), 2.14 [c] (1H. m)
11	178.6 (*s*)	-
12	14.1 (*q*)	1.15 (3H, s)
13	129.1 (*t*)	5.80 (1H, s), 6.48 (1H, s)
14	-	-
15	-	-

Multiplicities were determined by DEPT experiments.

[b, c] These values are interchangeable.

Results and Discussion

Fungal transformation of β-caryophyllene (44) by *Aspergillus niger*

Screening scale experiment showed that *Aspergillus niger* (ATCC 16404) has a capacity to transform compound **44** into its derivative **45** (Table-5) thus a large scale experiment was performed. Incubation of β-caryophyllene (**44**) with *A. niger* yielded metabolite **45** (Figure-8). Metabolite **45** was obtained with 7.78 % yield. A time course analysis of the transformation of **44** revealed that metabolite **45** was formed after 72 hour of incubation. The structure of metabolite **45** was deduced through comparative spectroscopic studies with β-caryophyllene (**44**).

In conclusion, biotransformation of β-caryophyllene (**44**) by *A. niger* provided an effective procedure for demethylation and lactonization. Compound **2** was reported for the first time, which can be used to synthesize new compounds with interesting biological activities.

Table-5: Screening scale biotransformation of β-caryophyllene (**44**) by various fungi.

Fungi	**Duration**	**Number of metabolites**
Aspergillus niger	7 days	1
Curvularia lunata	7 days	0
Cephalosporium aphidicola	7 days	0

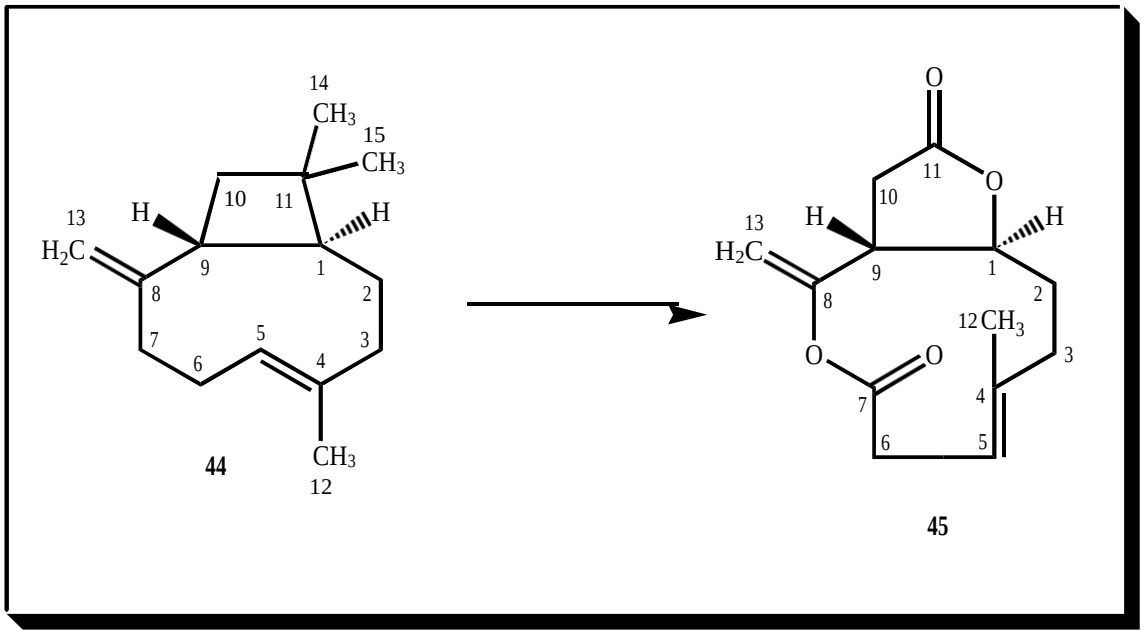


Fig. 8: Biotransformation of β-caryophyllene (**44**) by *Aspergillus niger*.

β-Caryophyllene (44) substrate

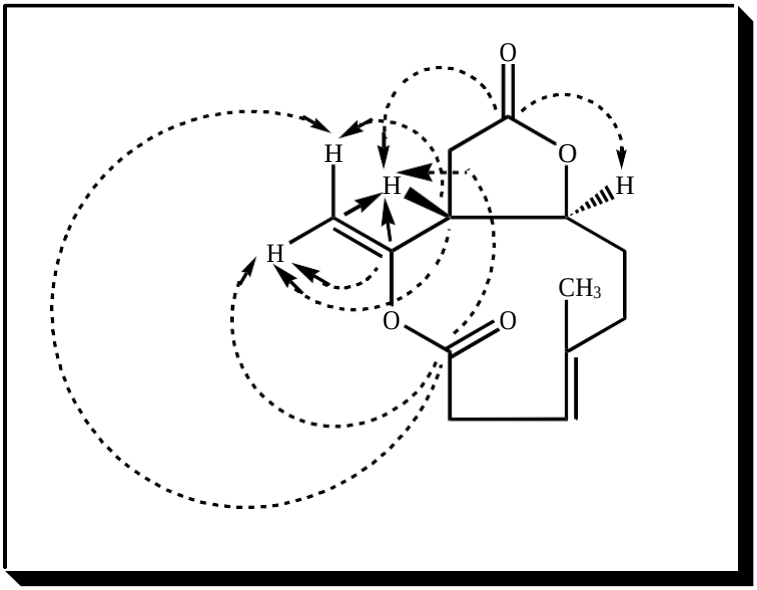
The ^{1}H- and ^{13}C-NMR data of β-carophyllene (**44**) (Table-2) was obtained and used to compare with ^{1}H- and ^{13}C-NMR data of metabolite **45**.

Metabolite 45 *(3aR,11aS,E)-9-Methyl-4-methylene-3a,4,7,10,11,11a- hexahydro-2H-furo[3,2-c]oxecine-2,6(3H)-dione* (**45**)

The HRESI-MS of metabolite **45** exhibited an $[M + H]^{+}$ at *m/z* 237.11038, corresponding to the formula $C_{13}H_{16}O_4$+H (calc. 237.11269), 32 a.m.u higher than **44**, indicating the incorporation of four oxygen atoms along with eliminations of two carbon atoms and eight hydrogen atoms. The IR spectrum showed the presence of carbonyl ester functionality (1752 cm^{-1}). The ^{1}H-NMR spectrum of metabolite **45** showed less number of hydrogen atoms, in comparison to the ^{1}H-NMR spectrum in **44**, while the ^{13}C-NMR spectrum showed two carbonyl carbons of ester functionality at 171.0 and 178.6, along with disappearance of C-7 methylene signal at 34.9, Ҁ

11 quaternary carbon signal at □ 33.9, and both identical C-14 and -15 methyl signals at □ 26.3, in comparison to compound **44**. Moreover the upfield shift of C-8 (□ 139.2) along with the downfield shift of C-1 (□ 72.4) suggested lactone formations in both rings of bicyclic system in compound **44**. HMBC spectrum of metabolite **45** showed correlations of H-9 (□ 3.39) with C-7 (□ 171.0), C-8 (□ 139.2), C-10 (□ 29.3), C-11 (□ 178.6) and C-13 (□ 129.3), while diastereotopic H_2-13 (□ 5.80 and 6.48) showed correlations with C-7 (□ 171.0), C-8 (□ 139.2) and C-9 (□ 47.0) (Figure-9) which supported lactone formations between C-6 and C-8, and between C-1 and C-10. COSY 45° Spectrum showed correlations of H-9 (□ 3.39) with H-1 (□ 3.79) and H_2- 10 (□ 1.92 and 2.14) (Figure-10) which further supported lactone formations between C-6 and C-8, and between C-1 and C-10. The structure of compound **45** was deduced as (3aR,11aS, E)-9-methyl-4-methylene-3a,4,7,10,11,11a-hexahydro-2H-furo[3,2- c]oxecine-2,6(3H)-dione. The scheme of proposed biotransformation to the metabolite **45** is shown in figure-11.

Fig. 9: HMBC (C → H) correlations in compound **45**.



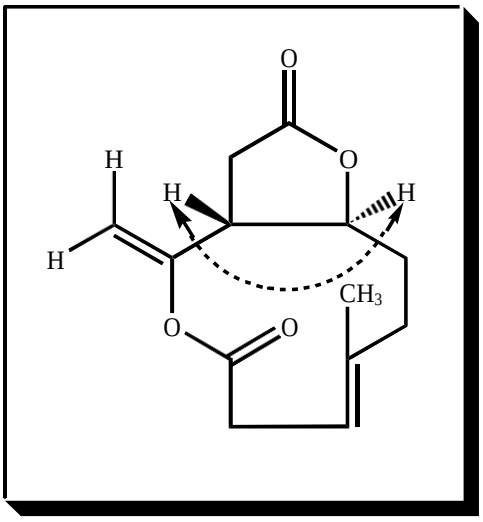


Fig. 10: COSY (↔) correlations in compound **45**.

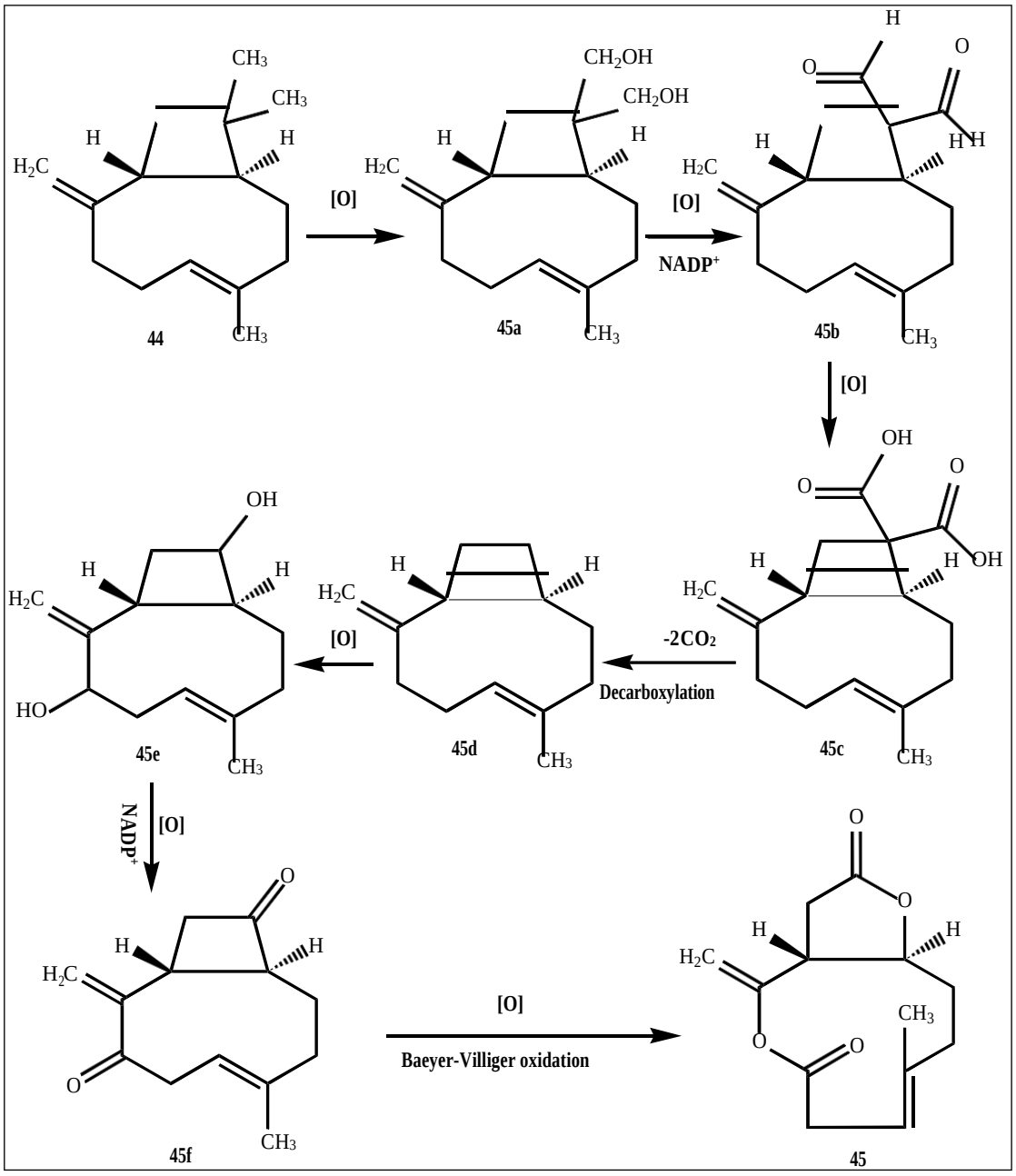


Figure-11: Scheme of proposed pathway to the metabolite **45**.

Anti-oxidant activity of β-caryophyllene (44) and the resulting metabolite 45 Metabolite **45** was tested for its antioxidant activity in comparison to **44** and ascorbic acid, and showed appreciable antioxidant activity against DPPH (Table-2). The DPPH scavenging activity of the β-caryophyllene (**44**) was concentration dependent. Metabolite **45** produced appreciable activity against DPPH.

β-Carotene bleaching (BCB)activity

The β-caryophyllene (**44**) was also effective in preventing the bleaching of β-carotene using linoleic acid. The IC_{50} of the β-caryophyllene (**44**) was 105.7, while the IC_{50} of rutin was 70.2 µg/ml. The IC_{50} values are mentioned in the table-6.

Table-6: The IC_{50} of DPPH radical and β-carotene bleaching assay of β- caryophyllene (**44**), metabolite **45** and the standard.

Sample	IC_{50} (DPPH radical) µg/ml	IC_{50} (β-carotene bleaching assay) µg/ml
β-Caryophyllene (**44**)	145.1 ± 1.5	105.7 ± 1.5
Metabolite **45**	> 1000	-
Ascorbic acid	5.5 ± 0.5	-
Rutin	-	70.2 ± 1.2

Values are expressed as mean ± SD (n=3)

4.0 Conclusion

Biotransformation of β-caryophyllene (**44**) by *A. niger* provided an effective procedure for the lactonization . Compound **45** was reported for the first time, and obtained in good yield which can be used to synthesize new compounds with interesting biological activities.

5.0 Future work

The new metabolite **45** will tested for its anti-microbial activity in comparison with the β-caryophyllene (**44**) and antibiotic as a reference. Cytotoxicity assay will be also performed.

6.0 References

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Summary in Arabic

الدراسات التحويلية الفطرية على β-Caryophyllene والتقييم البيولوجي للأيضات الناتجة

اعداد
قتيبة غازي ابراهيم الدليمي
المشرف
د.منال النجداوي

المشرف المساعد
د.محمد ياسين

ملخص

التحويل الحيوي لمركب β-carophyllene عن طريق فطر *Aspergillus niger* أنتج مركب واحد رئيس. المركب الناتج تم تعريفه عن طريق الطرق المطيافيه المختلفه وتم توثيقه هنا كمركب جديد. تم دراسة المركب الناتج كمضاد للأكسده مقارنة ب β-caryophyllene و ascorbic acid حيث وجد أن مركب الأيض الناتج لديه تأثير متوسط كمضاد للأكسده.

7.0 Supplementary materials

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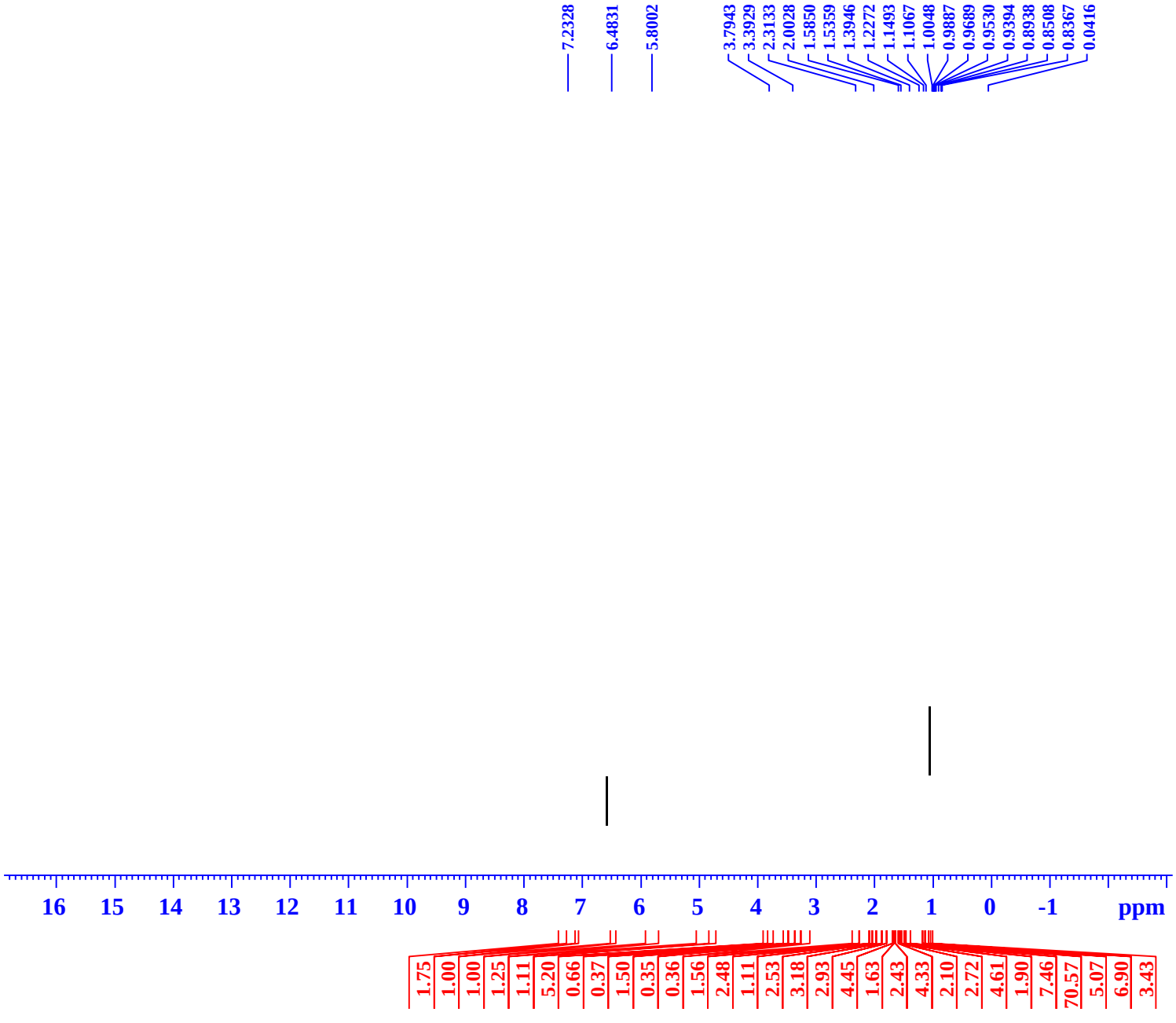
H-NMR



The University of Jordan
Faculty of Science
Department of Chemistry

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Operator: Rola Hassouneh
nmr500@ju.edu.jo

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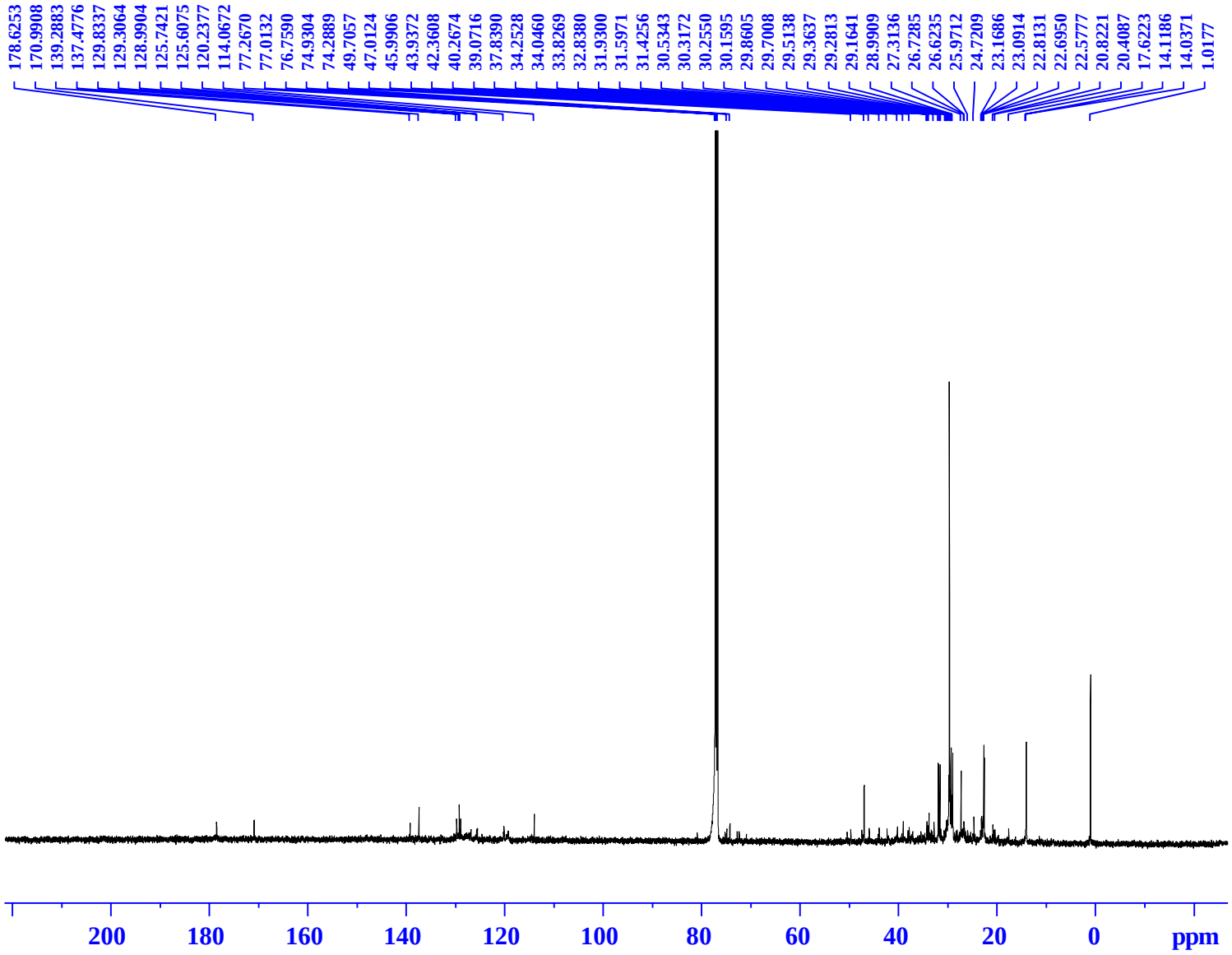

The University of Jordan
Faculty of Science
Department of Chemistry

Instrument Model:
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Operator: Rola Hassouneh
nmr500@ju.edu.jo

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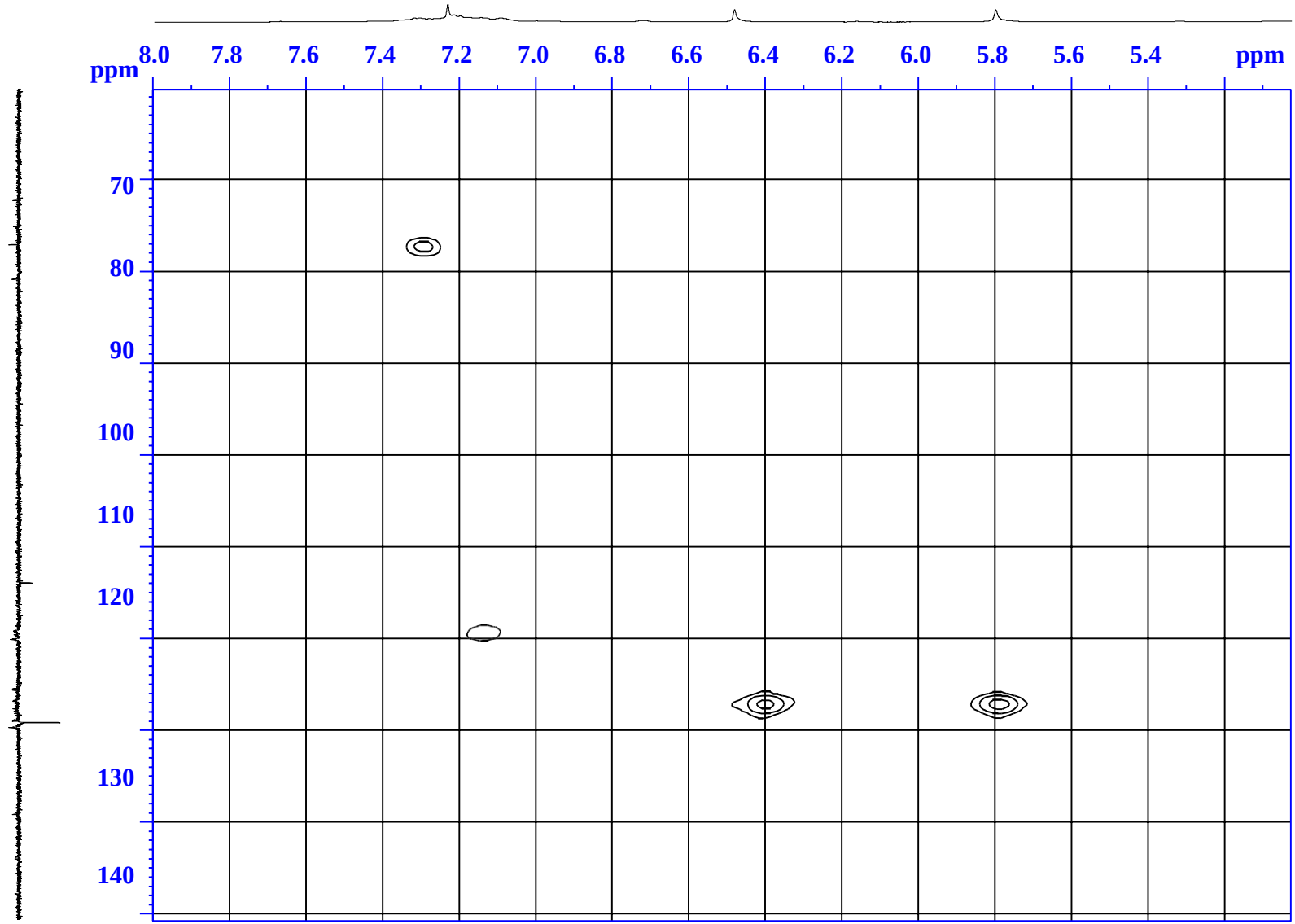

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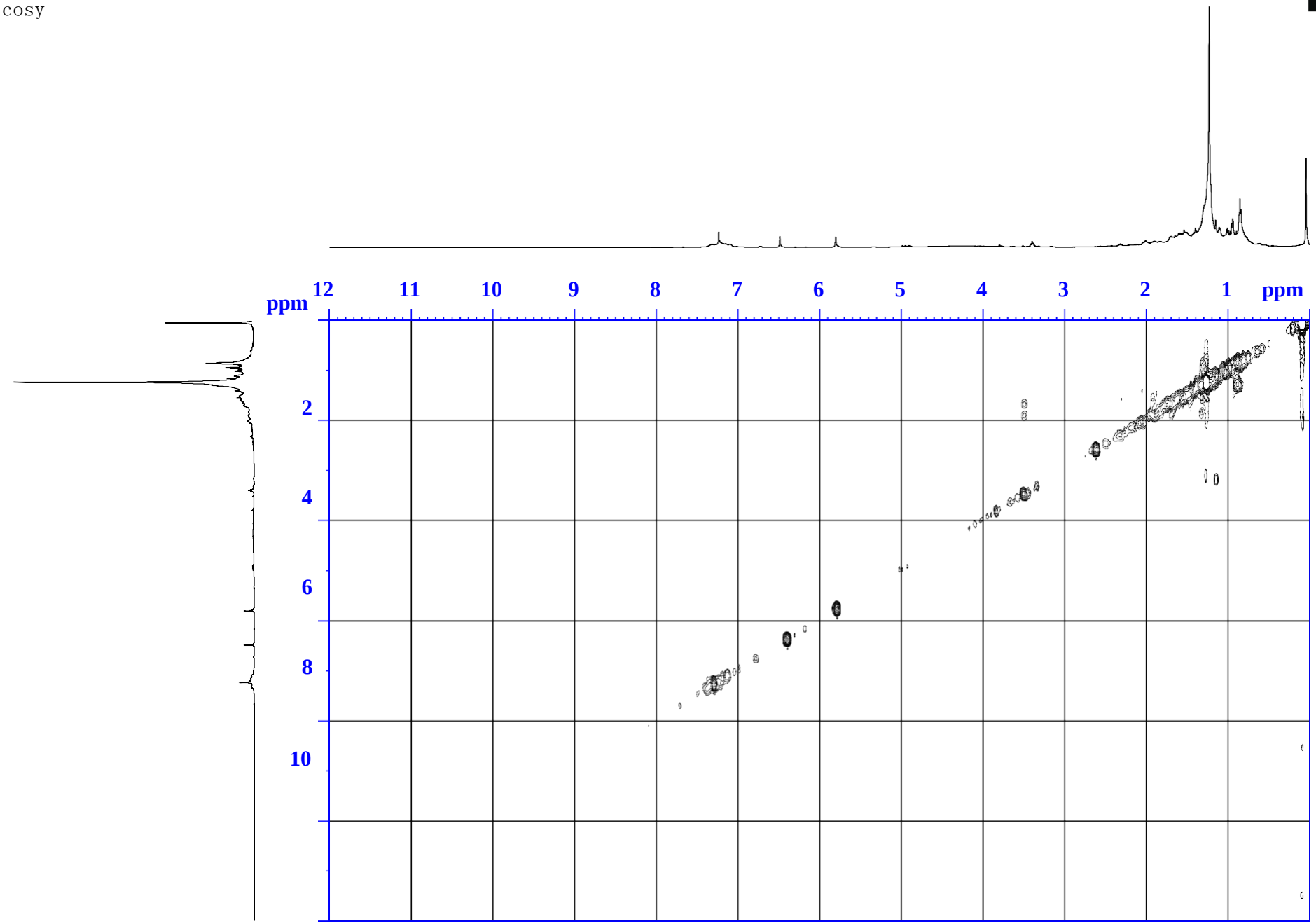

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AsBC-(23-28)
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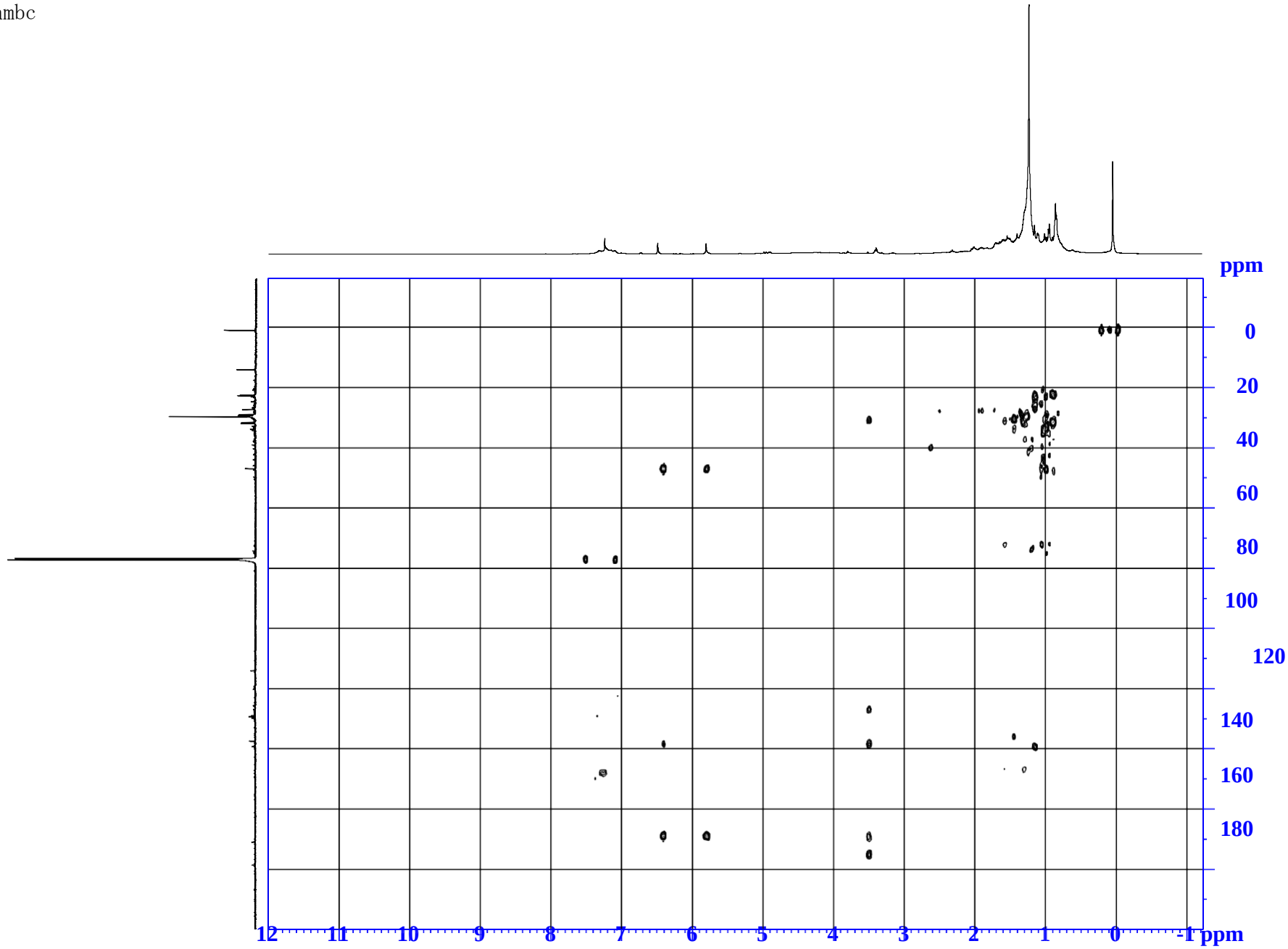

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D6 0.05000000 sec
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SFO2 125.7713681 MHz
NUC2 13C
P3 10.00 use
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GPZ1 50.00 %
GPNAM[2] SMSQ10.100
GPZ2 30.00 %
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PC 1.40

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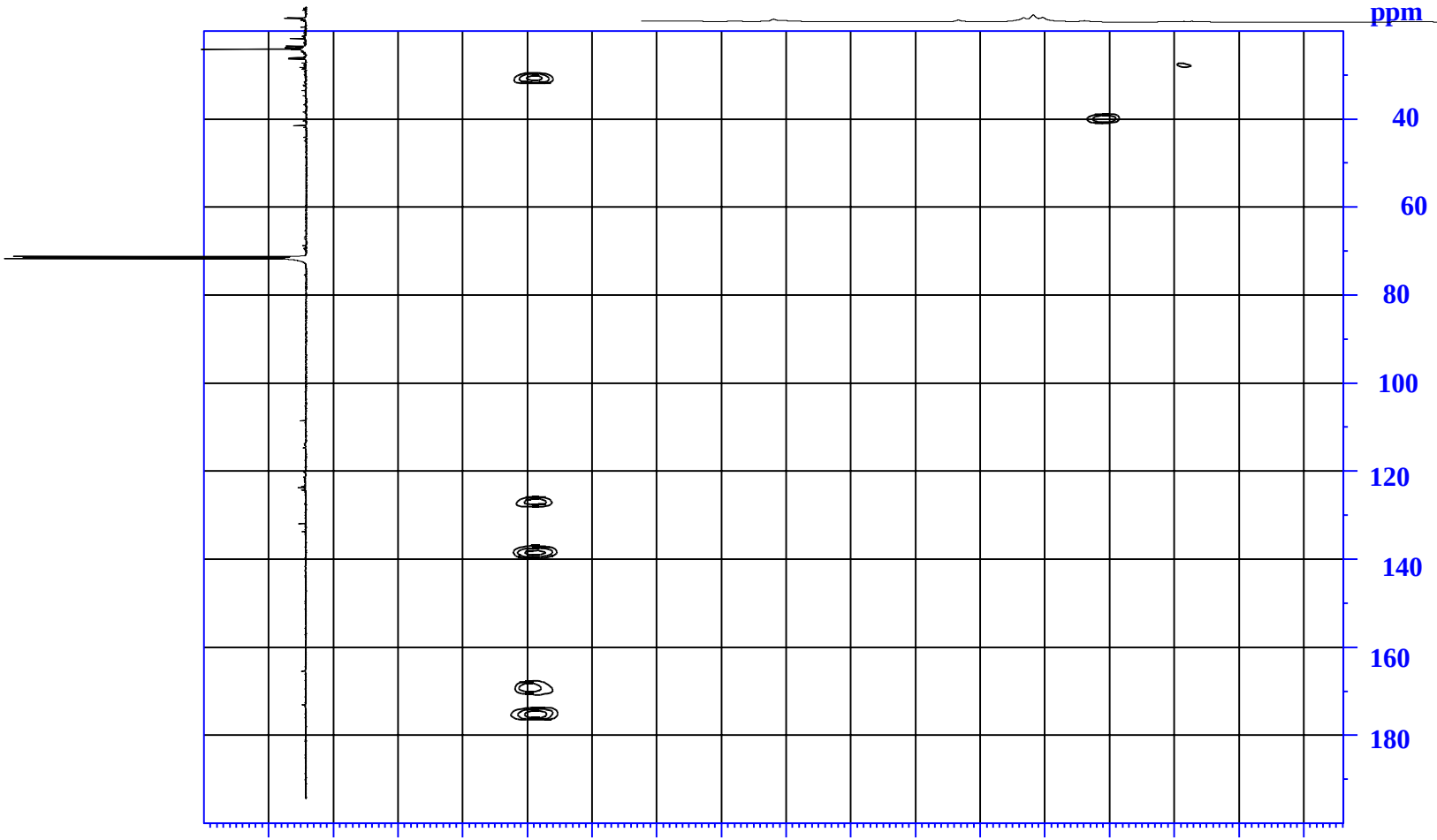
AsBC-(23-28)
hmbc



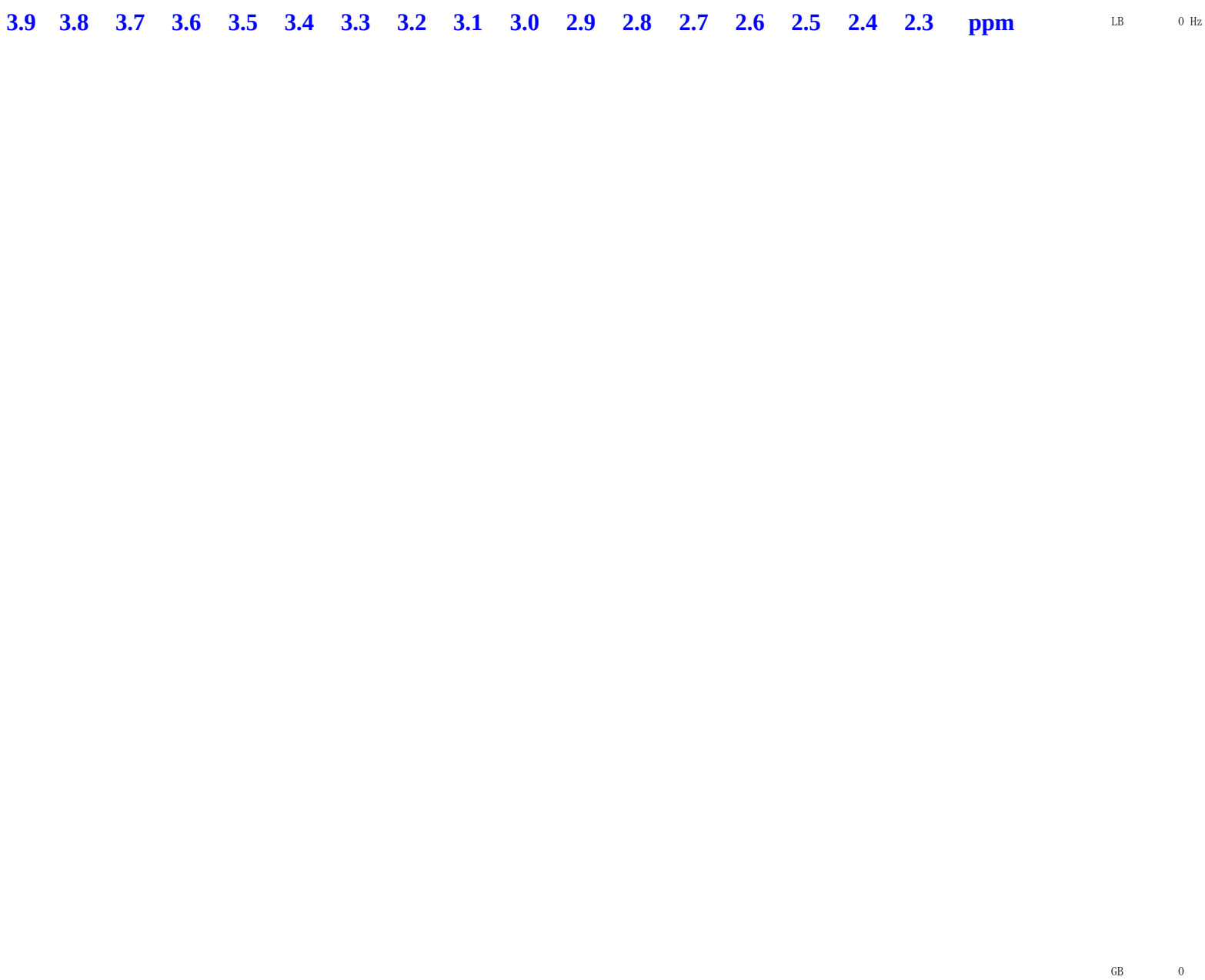
Current Data Parameters
NAME 20mar01jalal
EXPNO 1627
PROCNO 1

F2 - Acquisition Parameters
Date_ 20200603
Time 10.45 h
INSTRUM spect
PROBHD Z119470_0023 (
PULPROG hmbcgplpndqf
TD 2048
SOLVENT CDCl3
NS 32
DS 16
SWH 9973.404 Hz
FIDRES 9.739653 Hz
AQ 0.1026731 sec
RG 202.96
DW 50.133 use
DE 6.50 use
TE 299.0 K
CNST2 145.0000000
CNST13 10.0000000
D0 0.00000300 sec
D1 1.50000000 sec
D2 0.00344828 sec
D6 0.05000000 sec
D16 0.00020000 sec
IN0 0.00001600 sec
TDav 1
SFO1 500.1343701 MHz
NUC1 1H
P1 12.00 use
P2 24.00 use
PLW1 13.32299995 W
SFO2 125.7713681 MHz
NUC2 13C
P3 10.00 use
PLW2 96.27500153 W
GPNAM[1] SMSQ10.100
GPZ1 50.00 %
GPNAM[2] SMSQ10.100
GPZ2 30.00 %
GPNAM[3] SMSQ10.100
GPZ3 40.10 %
P16 1000.00 use

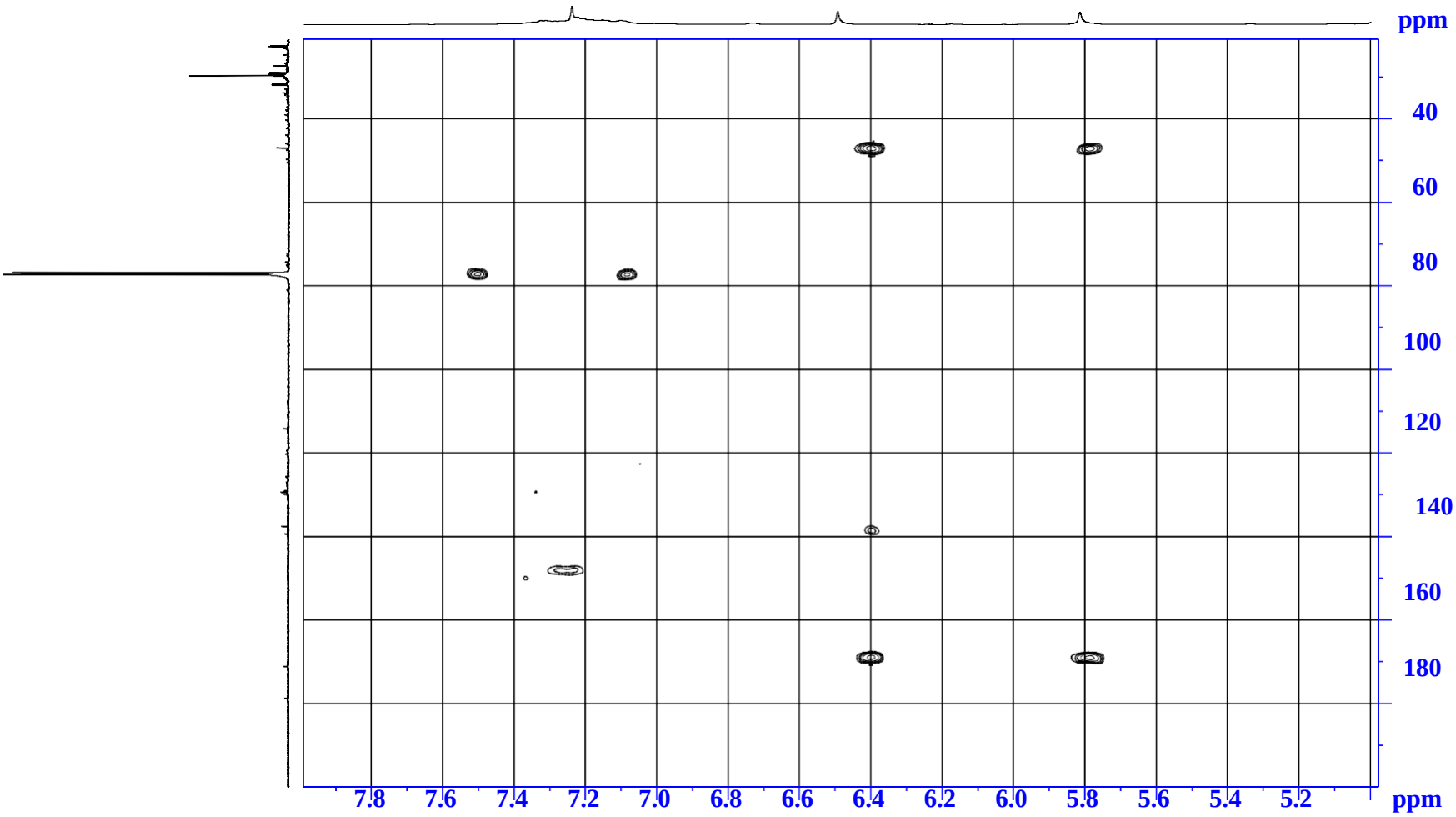
F1 - Acquisition parameters TD
128
SFO1 125.7714 MHz
FIDRES 488.281250 Hz
SW 248.467 ppm
FnMODE QF

F2 - Processing parameters
SI 2048
SF 500.1300000 MHz
WDW SINE
SSB 0
LB 0 Hz
GB 0
PC 1.40

F1 - Processing parameters SI
1024
MC2 QF
SF 125.7577890 MHz
WDW SINE
SSB 0
GB 0


3.9
3.8
3.7
3.6
3.5
3.4
3.3
3.2
3.1
3.0
2.9
2.8
2.7
2.6
2.5
2.4
2.3
ppm
LB
0 Hz
GB
0

AsBC-(23-28)
hmbc



Current Data Parameters
NAME 20mar01jalal
EXPNO 1627
PROCNO 1

F2 - Acquisition Parameters
Date_ 20200603
Time 10.45 h
INSTRUM spect
PROBHD Z119470_0023 (
PULPROG hmbcgplpndqf
TD 2048
SOLVENT CDCl3
NS 32
DS 16
SWH 9973.404 Hz
FIDRES 9.739653 Hz
AQ 0.1026731 sec
RG 202.06
DW 50.133 use
DE 6.50 use
TE 299.0 K
CNST2 145.0000000
CNST13 10.0000000
D0 0.00000300 sec
D1 1.50000000 sec
D2 0.00344828 sec
D6 0.05000000 sec
D16 0.00020000 sec
IN0 0.00001600 sec
TDav 1
SFO1 500.1343701 MHz
NUC1 1H
P1 12.00 use
P2 24.00 use
PLW1 13.32299995 W
SFO2 125.7713681 MHz
NUC2 13C
P3 10.00 use
PLW2 96.27500153 W
GPZ1 50.00 %
GPNAM[2] SMSQ10.100
GPZ2 30.00 %
GPNAM[3] SMSQ10.100
GPZ3 40.10 %
P16 1000.00 use

F1 - Acquisition parameters TD
128
SFO1 125.7714 MHz
FIDRES 488.281250 Hz
SW 248.467 ppm
FnMODE QF
F2 - Processing parameters

SI 2048
SF 500.1300000 MHz
WDW SINE
SSB 0
LB 0 Hz
GB 0
PC 1.40

F1 - Processing parameters SI
1024
MC2 QF
SF 125.7577890 MHz
WDW SINE
SSB 0

LB 0 Hz
GB 0